pharmaceutically acceptable carrier for administration of the composition in an amount and mode of administration effective to induce tolerance to EBV-associated immune responses.

Remarks

Claims 1-5, and 11-18 are pending in the application. Applicants appreciate the withdrawal of the objection to the specification for not referencing the priority applications and of the rejection of claims 1, 2, 3, 5, 11, 12, 13, and 16 under 35 U.S.C. § 102(b) over U.S. Patent No. 4,707,358 to Kieff, of claims 1, 2, 3, 5, 11, 12, 13, and 16 under 35 U.S.C. § 102(e) over U.S. Patent No. 5,726,286 to Alderson, of claims 1, 2, 3, 5, 11, 12, 13, and 16 under 35 U.S.C. § 102(e) over U.S. Patent No.5,679,774, to Wolf, of claims 1-5, 11, 12, 13, 14, and 16 under 35 U.S.C. § 102(b) over U.S. Patent No. 4,654,419 to Vaughan, of claims 14, 15, 17, and 18 under 35 U.S.C. § 103 over U.S. Patent No.4,654,419 to Vaughan in combination with Harley and James, J. Lab. Clin. Med., 126(6):509-516 (1995). The new issues raised by the Examiner are addressed in detail below.

Amendments to the Claims

Claim 1 has been amended so that there is proper antecedent basis by changing "vaccine" to "composition."

Claim 4 has been amended to correctly recite the appropriate SEQ ID NOs.

Claim 11 has been amended to correct an antecedent basis problem with the word "vaccine" by changing the word "vaccine" to "composition." Claim 11 was also amended to address the Examiner's concerns regarding 35 U.S.C. 112 second paragraph.

Claims 16 and 17 were amended to address an antecedent basis problem with the word "vaccine" by changing "vaccine" to "composition."

Support for new claims 27 and 28, which were suggested by the Examiner in the Office Action mailed, March 17, 1999 can be found at least in claim 8.

Pursuant to 37 C.F.R. §1.116(b), it is submitted that these amendments should be entered, as they do not introduce new matter or raise new issues that require a new search and they place the claims in condition for allowance or in better form for appeal.

Objection to the Information Disclosure Statement

Submitted with this Response is a Supplemental Information Disclosure Statement, PTO-Form 1449, listing the patents and publications that Applicant believes the Examiner has requested copies of. Applicants have also corrected the omission of the "publication year" for Hardgrave et al. and Kaufman et al. Applicants believe that the requirements under 37 C.F.R § 1.98 were met by the submission of the Information Disclosure Statement, mailed on January 13, 1999. Applicants, however, wish to facilitate the Examiners review of this Information Disclosure Statement, and therefore have, and are trying, to comply with the Examiner's wishes. Applicants appreciate the consideration of all of the patents and publications listed on the Information Disclosure Statement, mailed January 13, 1999.

Rejections under 35 U.S.C. § 112, second paragraph

Claims 4 and 11-18 were rejected under 35 U.S.C. § 112, second paragraph for allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter. This rejection is respectfully traversed if applied to the amended claims.

Claim 4 has been amended to recite the correct SEQ ID NOs as noted by the Examiner.

The Examiner asserted that the terms "individual at risk" and "symptoms associated with" in claim 11 were relative terms because "at risk' and 'symptoms associated with' are not defined by the claim or the specification . . . and one of ordinary skill would not . . .[understand] the scope of the invention." Claim 11 has been amended to facilitate prosecution by deleting the terms "individual at risk" and "symptoms associated with." These terms are not required for patentability, and therefore have been removed to address the Examiner's concerns.

Alternatively, if the present amendment is not entered both phrases, "individuals at risk" and "symptoms associated with," are definite. The "at risk" language and corresponding clinical criteria are well known to those skilled in the field of treating autoimmune disorders, and support for this can be found in the application. For example, it is well known that individuals at risk can be assessed by virtue of family studies or genetic analysis (for example, see discussion regarding genetics on page 31 of the application), age; sex, and ethnic background (for example, it is well established that lupus is more common in young female adults than in other groups); and other factors. It is equally well known that certain symptoms can be assessed to diagnose autoimmune disease, as well as its severity. An example is by measurement of autoantibodies (see page 12,

for example, and Example 1 beginning at page 27.) Infection with EBV is a risk factor, but it certainly is not the only risk factor that one of ordinary skill in the art would consider, or which is discussed in the application.

Furthermore, the symptoms associated with autoimmune diseases are well established and well known to those of skill in the art as the Examiner is quite familiar with, based on our interview. Applicants are not required to include information known to the skilled artisan. In fact, the application preferably omits such information. *Spectra-Physics, Inc. v. Coherent, Inc.*, 827 F.2d 1524, 3 USPQ2d 1737 (Fed. Cir. 1987). The person of ordinary skill in the art knows and understands the symptoms associated with autoimmune diseases. In fact, this is how the diseases were and are classified, by their symptoms. The fact that different autoimmune diseases exist, as pointed out by the Examiner, is *prima facie* proof that the diseases have unique fingerprints and that these fingerprints for diagnosis are well known to those of skill in the art.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 1-5 and 11-18 were rejected under 35 U.S.C. § 112, first paragraph for allegedly containing subject matter which was not described in the specification in such a way as to enable one of skill in the art to make and use the claimed subject matter. This rejection is respectfully traversed.

Law of requirements for pharmaceutical inventions

An invention must have utility. This requirement can be found in U.S.C. § 101 which states," Whoever invents or discovers any new and *useful* process or . . . composition of matter . . .

may obtain a patent . . ." (emphasis added). This requirement is also implicitly found in 35 U.S.C. § 112 which requires the specification to provide a written description for "making and *using*" the claimed subject matter.

Whether the utility requirement comes from 35 U.S.C. § 101 or 35 U.S.C. § 112, the standard to be applied is the same. *Ex parte Maas*, 14 USPQ2d 1762, 9 USPQ2d 1746, 1747 (Bd. Pat. App. & Int'f 1987). The *Maas* court stated, "the issue under 35 U.S.C. § 112 relating to an enabling disclosure is subsumed within the issue under 35 U.S.C. § 101 relating to patentable utility." Any analysis of a claim under 35 U.S.C. § 112, first paragraph relating to the use of the claimed subject matter, need only meet the standards of the utility requirement of 35 U.S.C. § 101 because if the claimed subject matter meets the utility requirement it is presumed to meet the enablement requirement of use.

To meet the utility requirement the invention must simply have a "practical utility" in the "real world sense." (*Nelson v. Bowler*, 626 F.2d 853, 856 (CCPA, 1980)). Any use which gives immediate benefit to the public is sufficient to be a "practical utility". *Id.* at 856. It is clear that for an invention to have "practical utility" it must be operative. However, to fail the utility requirement the claimed subject matter must be "totally incapable of achieving a useful result."[1/] (*Brooktree Corp v. Advanced Micro Devices. Inc.*, 977 F.2d 1555 (Fed. Cir. 1992). An assertion of utility is

[1/]See also *E.I. du Pont De Nemours and Co. v. Berkley and Co.*, 620 F.2d 1247, 1260 n.17, 205 USPQ 1, 10 n.17 (8th Cir. 1980) ("In short, the defense of non-utility cannot be sustained without proof of total incapacity.").

sufficient to meet the utility requirement unless the assertion is "incredible in the light of the art or factually misleading." (*In re Citron*, 325 F.2d 1389 (CCPA, 1963)).

The standard for utility does not change if the subject matter is pharmaceutical or therapeutic in nature. (*In re Chilowsky*, 229 F.2d 457, 461-2 (CCPA 1956)). "Knowledge of pharmacological activity is an obvious benefit to the public. . . . [A]dequate proof of any such activity constitutes a showing of practical utility" (*Nelson v. Bowler*, 626 F.2d 853, 856 (CCPA, 1980)). The Federal Circuit held that adequate proof of a pharmacological activity can be obtained by merely providing *in vitro* data which are suggestive of an activity *in vivo*. (*Cross v. Iizuka*, 753 F.2d 1040 (CAFC, 1985). "Successful *in vitro* testing . . . [will lead to] . . . *in vivo* testing . . . thereby providing an immediate benefit to the public, analogous to the benefit provided by the showing of an *in vivo* utility." *Id.* at 1051. Furthermore, data obtained from animal models clearly is adequate proof. *In re Krimmel* 292 F.2d 948 (CCPA, 1961). The *Krimmel* court stated, "one who has taught the public that a compound exhibits some desirable pharmaceutical property in a standard experimental animal has made a significant contribution to the art even though it may eventually appear that the compound is without value in the treatment of humans." *Id.* at 953.

Future testing in animals and future testing in humans, even if extensive, does not prevent a specification from meeting the utility requirement. The Court stated in *In re Brana*, "Usefulness in Patent law and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development." (*In re Brana*, 51 F.3d 1560, 1568 (Fed. Cir. 1995)). If the subject matter covered by pharmaceutical inventions requires future research and

development, even after conception and constructive reduction to practice, when then is the utility requirement met? The Federal Circuit has answered this question: "The stage at which an invention in this field becomes useful [i.e. enabled with respect to use requirement] is *well before* it is ready to be administered to humans." (emphasis added) *Id.* at 1568.

The law does not explicitly state what is required to meet the utility requirement for any given pharmacological use because an analysis of utility is a fact based decision. (*Ratheon v. Roper*, 724 F.2d 956). The law is explicitly clear, however, as to what pharmaceutical utility does not require. Pharmaceutical utility does not require human testing.[2/] Pharmaceutical utility does not require animal testing.[3/] Pharmaceutical utility does not require a showing of therapeutic safety.[4/] Most importantly, pharmaceutical utility does not require a showing of efficacy.[5/]

Claims 1-5 and 11-18 are fully enabled as required by the Federal Circuit

The current rejection is a use rejection of claims 1-5 and 11-18. Claims 1-5 are drawn to compositions comprising a modified Epstein-Barr virus or a modified component thereof, wherein one or more structures of the Epstein-Barr virus are removed or altered to decrease the potential that the composition will induce an autoimmune disorder, in a pharmaceutically

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[2/] *In re Jolles*, 628 F.2d 1322 (CCPA, 1980); *In re Krimmel*, 292 F.2d 948 (CCPA, 1961); *Cross v. Iizuka*, 753 F.2d 1040 (1985); and *In re Brana* 51 F.3d 1560 (Fed. Cir. 1995).
[3/] *In re Krimmel*, 292 F.2d 948 (CCPA, 1961) and *Cross v. Iizuka*, 753 F.2d 1040 (1985).
[4/] *In re Brana* 51 F.3d 1560 (Fed. Cir. 1995) and *In re Irons*, 340 F.2d 974, 978 (CCPA 1965).
[5/] See *In re Sichert*, 566 F.2d 1154, 196 USPQ 209 (1977); In re *Hartop*, 311 F.2d 249, 135 USPQ 419 (CCPA 1962); *In re Anthony*, 414 F.2d 1383, 162 USPQ 594 (CCPA 1969); *In re Watson*, 517 F.2d 465, 186 USPQ 11 (CCPA 1975); *In re Krimmel*, 292 F.2d 948, 130 USPQ 215 (CCPA 1961); *Ex parte Jovanovics*, 211 USPQ 907 (Bd. Pat. App. & Inter. 1981).

acceptable carrier for administration of the virus or viral component in an amount and mode of administration effective to alleviate or prevent symptoms associated with the autoimmune disorders. Claims 11-18 are drawn to methods comprising administering to an individual who has an autoimmune disorder induced by infection with Epstein-Barr virus, a composition comprising a killed or attenuated Epstein-Barr virus or a component thereof, or modifications thereof wherein one or more structures of the Epstein-Barr virus are removed or altered to decrease the potential that the vaccine will induce an autoimmune disorder, in a pharmaceutically acceptable carrier for administration of the virus or viral component in an amount and mode of administration effective to alleviate or prevent the autoimmune disorders.

Of particular importance is the fact that neither claims 1-5, claims 11-18, or claims 27 and 28 require a specified level of efficacy, nor do they require the cure of any autoimmune disease. The Examiner has made it clear in the Office Action dated March 17, 1999, that "No evidence has been set forth which shows the lessening of any symptom of an autoimmune disease by the administration of a composition of the invention." The Examiner also states, "The specification does not set forth any examples wherein the administration of the elected composition in an accepted animal model is able to successfully "alleviate" an already existing autoimmune disease" and "There are no experiments which challenge vaccinated animals with live unattenuated EBV such that the prevention of the autoimmune disease is shown." Applicants are not required to show or provide the types of data that the Examiner demands. The efficacy or the extent of therapeutic effectiveness is to be addressed at the FDA, not the PTO.

The Federal Circuit is clear (see above) that the time that pharmaceuticals are ready for patenting is well before they are ready for use or treatment in a human. There is absolutely no requirement one provide animal model data.

Applicants are required to show that the claimed compounds or methods are likely to have the pharmaceutical utility and the Federal Circuit has indicated that *in vitro* data are sufficient for this if it is "suggestive of an activity *in vivo*." (*Cross v. Iizuka*, 753 F.2d 1040 (CAFC, 1985)). The data provided in the application and verified by the experiments described in the Declaration under C.F.R 1.132 by Dr. Harley clearly indicate that the claimed compounds are likely to have an effect on the course of autoimmune diseases. Autoimmune diseases are associated with the production of antibodies to a variety of epitopes and the use of these epitopes for desensitization or the use of vaccines absent the epitopes is clearly indicated by the *in vitro* data linking the autoantibodies of autoimmune diseases and the epitopes of the claimed subject matter. The present application clearly establishes the connection between the epitopes and the autoimmune diseases of the claims.

Not withstanding the above Applicants have provided a number of references which indicate that the *in vitro* binding data of epitopes involved in autoimmune-type diseases are predictive of *in vivo* use (which are enclosed). For example, Nicholson et al. present data that indicate a slightly mutated epitope of the proteolipid protein of myelin acts as an antagonist of the T cell receptor and blocks the binding of the epitope *in vitro* and functions *in vivo* (Nicholson et al., "A T cell receptor antagonist peptide induces T cells that mediate bystander suppression and prevent

autoimmune encephalomyelitis induced with multiple myelin antigens" Proc. Natl. Acad. Sci. U S A. 1997 Aug 19;94(17):9279-84). While the *in vivo* mechanisms were not fully elucidated, it was unequivocal that the treatment of the peptide halted the destruction of myelin in mice which is caused by an autoimmune attackon the myelin. Furthermore, Gautam et al. have shown that the herpesvirus *Saimiri* contains small epitopes which when injected into a mouse cause Experimental Autoimmune Encephalomyelitis (EAE) indicating that small epitopes can and do have effects *in vivo*. (Gautam et al., "A viral peptide with limited homology to a self peptide can induce clinical signs of experimental autoimmune encephalomyelitis" J Immunol. 1998 Jul 1;161(1):60-4) Lastly, Vandenbark et al., showed that vaccinations with epitopes related to EAE and Multiple Sclerosis caused protective responses to these diseases *in vivo* (Vandenbark et al., "Effects of vaccination with T cell receptor peptides: epitope switching to a possible disease-protective determinant of myelin basic protein that is cross-reactive with a TCR BV peptide." Immunol Cell Biol. 1998 Feb;76(1):83-90).

These publications from 1998 and 1997 support the claims of the present application which claims priority to 1993. There can be little doubt that the methods and compositions of the present claims are fully enabled by the specification.

The Examiner has indicated that claims drawn to the specific epitopes for induction of tolerance would be allowable. Applicants agree and have added claims 27 and 28 to specifically address this. In addition, however, EBV proteins lacking the antigenic epitopes are also fully enabled. Again the standard is whether there is *in vitro* data sufficient so that it is "suggestive of

an activity *in vivo*." (*Cross v. Iizuka*, 753 F.2d 1040 (CAFC, 1985)). Clearly the direct correlation of binding and response to a given epitope indicates that the removal of the epitope will lessen that response. Applicants have provided extensive data indicating that the epitopes are associated with the immunogenic autoimmune response and therefore, the removal of these epitopes from the presenting peptides would be undeniably expected to lessen the antigenic response to those presenting peptides.

Allowance of all claims 1-5, 11-18, and 27-28 as amended, is earnestly solicited. All claims as pending upon entry of this amendment are attached in an appendix for the convenience of the examiner.

Respectfully submitted,

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APPENDIX: Pending claims

1. (Amended) An immunogenic composition for alleviating or preventing symptoms of autoimmune disorders induced by infection with Epstein-Barr virus comprising

a modified Epstein-Barr virus or a modified component thereof, wherein one or more structures of the Epstein-Barr virus are removed or altered to decrease the potential that the composition will induce an autoimmune disorder, in a pharmaceutically acceptable carrier for administration of the virus or viral component in an amount and mode of administration effective to alleviate or prevent symptoms associated with the autoimmune disorders.

2. (Amended) The composition of claim 1 comprising modified Epstein-Barr virus components.

3. (Amended) The composition of claim 1 wherein the component of Epstein-Barr virus is selected from the group consisting of peptides or proteins expressed from recombinant DNA or RNA with sequence identity to Epstein-Barr virus, viral DNA or RNA, and carbohydrate components of the Epstein-Barr virus.

4. (Twice amended) The composition of claim 1 wherein the Epstein-Barr virus comprises the nuclear antigen 1 protein not including a peptide sequence selected from the group consisting of PPPGRRP (SEQ ID. NO:1), GRGRGRGG (SEQ ID NO: 2) and RGRGREK (SEQ ID NO: 3).

5. The composition of claim 1 in a pharmaceutical carrier for administration by injection.

11. (Amended) A method for preventing or alleviating autoimmune disorders induced by infection with Epstein-Barr virus comprising

administering to a individual at risk of developing, or who has been identified as having symptoms associated with, an autoimmune disorder induced by infection with Epstein-Barr virus,

a composition comprising a killed or attenuated Epstein-Barr virus or a component thereof, or modifications thereof wherein one or more structures of the Epstein-Barr virus are removed or altered to decrease the potential that the vaccine will induce an autoimmune disorder, in a pharmaceutically acceptable carrier for administration of the virus or viral component in an amount and mode of administration effective to alleviate or prevent the autoimmune disorders.

12. (Amended) The method of claim 11 wherein the composition comprises modified Epstein-Barr virus components.

13. The method of claim 11 wherein the component of Epstein-Barr virus is selected from the group consisting of peptides or proteins expressed from recombinant DNA or RNA with

sequence identity to Epstein-Barr virus, viral DNA or RNA, and carbohydrate components of the Epstein-Barr virus.

14. (Amended) The method of claim 11 wherein the Epstein-Barr virus comprises the nuclear antigen 1 protein not including a peptide sequence selected from the group consisting of PPPGRRP (SEQ ID NO:1), GRGRGRGG (SEQ ID NO:2) and RGRGREK (SEQ ID NO:3).

15. (Amended) The method of claim 11 wherein the individual has symptoms of or is at risk of developing an autoimmune disorder selected from the group consisting of systemic lupus erythematosus, Sjogren's syndrome, rheumatoid arthritis, juvenile onset diabetes mellitus, Wegener's granulomatosis, inflammatory bowel disease, polymyositis, dermatomyositis, multiple endocrine failure, Schmidt's syndrome, autoimmune uveitis, Addison's disease, adrenalitis, primary biliary cirrhosis, Graves' disease, thyroiditis, Hashimoto's thyroiditis, autoimmune thyroid disease, pernicious anemia, lupoid hepatitis, demyelating diseases, multiple sclerosis, subacute cutaneous lupus erythematosus, hypoparathyroidism, Dressler's syndrome, myasthenia gravis, autoimmune thrombocytopenia, idiopathic thrombocytopenic purpura, hemolytic anemia, autoimmune hemolytic anemia, pemphigus vulgaris, pemphigus, bullous pemphigoid, dermatitis herpetiformis, alopecia areata, autoimmune cystitis, pemphigoid, scleroderma, progressive systemic sclerosis, CREST syndrome (calcinosis, Raynaud's esophageal dysmotility, sclerodactyly, and telangiectasia), adult onset diabetes mellitus (Type II diabetes), male or female autoimmune infertility, ankylosing spondylitis, ulcerative colitis, Crohn's disease, mixed connective tissue disease, polyarteritis nodosa, systemic necrotizing vasculitis, juvenile onset rheumatoid arthritis, glomerulonephritis, atopic dermatitis, atopic rhinitis, Goodpasture's syndrome, Chagas' disease, sarcoidosis, rheumatic fever, asthma, recurrent abortion, anti-phospholipid syndrome, farmer's lung, erythema multiforme, postcardotomy syndrome, Cushing's syndrome, autoimmune chronic active hepatitis, bird-fancier's lung, allergic encephalomyelitis, toxic necrodermal lysis, alopecia, Alport's syndrome, alveolitis, allergic alveolitis, fibrosing alveolitis, interstitial lung disease, erythema nodosum, pyoderma gangrenosum, transfusion reaction, chronic fatigue syndrome, fibromyalgia, Takayasu's arteritis, Kawasaki's disease, polymyalgia rheumatica, temporal arteritis, giant cell arteritis, Sampter's syndrome (triaditis also called, nasal polyps, eosinophilia, and asthma), Behcet's disease, Caplan's syndrome, dengue, encephalomyositis, endocarditis, myocarditis, endomyocardial fibrosis, endophthalmitis, erythema elevatum et diutinum, psoriasis, erythroblastosis fetalis, fascitis with eosinophilia, Shulman's syndrome, Felty's syndrome, filariasis, cyclitis, chronic cyclitis, heterochromic cyclitis, Fuch's cyclitis, IgA nephropathy, Henoch-Schonlein purpura, glomerulonephritis, cardiomyopathy, post vaccination syndromes, Hodgkin's and non-Hodgkin's lymphoma, renal cell carcinoma, Eaton-Lambert syndrome, relapsing polychondritis.

16. (Amended) The method of claim 11 wherein the vaccine is administered prior to infection with Epstein-Barr virus.

17. (Amended) The method of claim 11 wherein the vaccine is administered to an individual who has or has previously had an infection with Epstein-Barr virus.

18. (Amended) The method of claim 11 wherein the autoimmune disorder is systemic lupus erythematosus.

27 (New) An immunogenic composition comprising a molecule selected from the group consisting of PPPGRRP (SEQ ID NO:1), GRGRGRGG (SEQ ID NO:2), RGRGREK (SEQ ID NO:3), GAGAGAGAGAGAGAGAGAGAGA (SEQ ID NO:7), GPQRRGGDNHGRGRGRGRGGGRPG (SEQ ID NO:13), GGSGSGPRHRDGVRRPQKRP (SEQ ID NO:14), RPQKRPSC (SEQ ID NO:15), QKRPSCIGCKGTHGGTG (SEQ ID NO:16), GTGAGAGARGRGG (SEQ ID NO:17), SGGRGRGG (SEQ ID NO:18), RGGSGGRRGRGR (SEQ ID NO:19), RARGRGRGRGEKRPRS (SEQ ID NO:20), SSSSGSPPRRPPPGR (SEQ ID NO:21), RPPPGRRPFFHPVGEADYFEYHQEG (SEQ ID NO:22), PDVPPGAI (SEQ ID NO:23), PGAIEQGPA (SEQ ID NO:24), GPSTGPRG (SEQ ID NO:25), GQGDGGRRK (SEQ ID NO:26), DGGRRKKGGWFGKHR (SEQ ID NO:27), GKHRGQGGSN (SEQ ID NO:28), GQGGSNPK (SEQ ID NO:29), NPKFENIA (SEQ ID NO:30), RSHVERTT (SEQ ID NO:31), VFVYGGSKT (SEQ ID NO:32), GSKTSLYNL (SEQ ID NO:33), GMAPGPGP (SEQ ID NO:34), PQPGPLRE (SEQ ID NO:35), CNIRVTVC (SEQ ID NO:36), RVTVCSFDDG (SEQ ID NO:37), and PPWFPPMVEG (SEQ ID NO:38), wherein the composition is in a pharmaceutically acceptable carrier for administration of the composition in an amount and mode of administration effective to induce tolerance to EBV-associated immune responses.

28 (New) A method comprising administering to a individual a composition comprising a molecule selected from the group consisting of PPPGRRP (SEQ ID NO:1), GRGRGRGG (SEQ ID NO:2), RGRGREK (SEQ ID NO:3), GAGAGAGAGAGAGAGAGAGAGA (SEQ ID NO:7), GPQRRGGDNHGRGRGRGRGGGRPG (SEQ ID NO:13), GGSGSGPRHRDGVRRPQKRP (SEQ ID NO:14), RPQKRPSC (SEQ ID NO:15), QKRPSCIGCKGTHGGTG (SEQ ID NO:16), GTGAGAGARGRGG (SEQ ID NO:17), SGGRGRGG (SEQ ID NO:18), RGGSGGRRGRGR (SEQ ID NO:19), RARGRGRGRGEKRPRS (SEQ ID NO:20), SSSSGSPPRRPPPGR (SEQ ID NO:21), RPPPGRRPFFHPVGEADYFEYHQEG (SEQ ID NO:22), PDVPPGAI (SEQ ID NO:23), PGAIEQGPA (SEQ ID NO:24), GPSTGPRG (SEQ ID NO:25), GQGDGGRRK (SEQ ID NO:26), DGGRRKKGGWFGKHR (SEQ ID NO:27), GKHRGQGGSN (SEQ ID NO:28), GQGGSNPK (SEQ ID NO:29), NPKFENIA (SEQ ID NO:30), RSHVERTT (SEQ ID NO:31), VFVYGGSKT (SEQ ID NO:32), GSKTSLYNL (SEQ ID NO:33), GMAPGPGP (SEQ ID NO:34), PQPGPLRE (SEQ ID NO:35), CNIRVTVC (SEQ ID NO:36), RVTVCSFDDG (SEQ ID NO:37), and PPWFPPMVEG (SEQ ID NO:38), wherein the composition is in a pharmaceutically acceptable carrier for administration of the composition in an amount and mode of administration effective to induce tolerance to EBV-associated immune responses.

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Effects of vaccination with T cell receptor peptides: Epitope switching to a possible disease-protective determinant of myelin basic protein that is cross-reactive with a TCR BV peptide

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Summary Immunization of Lewis rats with guinea-pig myelin basic protein (Gp-MBP) induced T cell responses to primary and secondary encephalitogenic determinants, as well as to a third non-encephalitogenic epitope, residues 55–69. This sequence is of interest due to its protective activity against experimental autoimmune encephalomyelitis. Protection involved induction of MBP-55–69-specific T cells expressing cross-reactive TCR BV8S6 genes that activated regulatory T cells specific for TCR BV8S2 determinants expressed on encephalitogenic T cells. We here present and discuss new evidence suggesting a possible immunological cross-reactivity between the protective Gp-MBP-55–69 peptide and the regulatory BV8S2-39–59 peptide. This cross-reactivity, which may also occur between the human MBP-55–74 peptide and the BV12S2-38–58 sequence, has potentially important implications for human diseases such as multiple sclerosis.

Key words: cross-reactivity, experimental autoimmune encephalomyelitis, multiple sclerosis, myelin basic protein, regulatory T cells, T cell receptor peptides.

Introduction

T cell recognition of naturally processed TCR determinants expressed with self-MHC molecules on pathogenic autoreactive Th1 cells stimulates an effective peripheral regulatory mechanism involving production and release of suppressive cytokines, including IL-10.[1] In animal models, the induction of encephalitogenic T cells expressing disease-associated V genes during development of experimental autoimmune encephalomyelitis (EAE) naturally spawns a compensatory wave of regulatory T cells specific for V gene determinants that contributes to spontaneous recovery from EAE.[2] Vaccination with TCR peptides boosts the frequency and Th2 lymphokine production of the TCR-specific T cells, resulting in a rapid inactivation of Th1 cells specific for immunodominant epitopes.[3–5] Moreover, TCR vaccination reduced the frequency of myelin basic protein (MBP)-specific T cells, and caused a switch in T cell specificity away from the pathogenic MBP-72–89 determinant t wards other MBP epitopes.[6] Given prior to disease onset, vaccination with TCR peptides can prevent EAE: given after onset of clinical signs, TCR vaccination can reverse clinical progression and speed recovery.[7]

Sustained immunization with multideterminant organ-specific autoantigens such as MBP induces T cells specific for other MBP determinants or other central nervous system (CNS) antigens that may themselves be encephalitogenic.[8,9] This phenomenon, termed epitope spreading, may contribute to relapsing EAE. Alternatively, T cells may appear that are specific for minor, less dominant determinants. In previous reports, we have described one such determinant, guinea-pig (Gp)-MBP residues 55–69, that is protective against EAE in Lewis rats.[10] One mechanism for protection involved the expression of immunologically cross-reactive TCR V gene sequences by MBP-55–69-specific T cells.[11] We here present new evidence suggesting that there is sequence homology and immunological cross-reactivity between the rat BV8S2 CDR2 and Gp-MBP-55–69 sequences. Moreover, a similar homology exists between the human BV12 CDR2 and MBP-55–69. This raises the intriguing possibility that the MBP-55–69 determinant acts as a protective epitope in patients who are naturally sensitized to MBP, including those with extensive demyelination as occurs in multiple sclerosis (MS).

Recognition of MBP-55–69 in EAE

In the Lewis rat model of EAE, one of the most potent encephalitogens is Gp-MBP. The immunodominant determinant of Gp-MBP includes residues 72–89, which induces a high frequency of encephalitogenic Gp-MBP-specific T cells that can also recognize the rat (Rt) 72–89 sequence that differs only at position 80 by a Thr for Ser substitution.[12] T cells specific for Gp-MBP-72–89 typically express the AV2 and BV8S2 genes and are restricted by the RT-1B

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I-A molecule.[13–14] Sustained sensitization with Gp- or Rt-MBP also induces T cells specific for a secondary encephalitogenic determinant, residues 87–99, that is identical in both species.[15–16] T cells specific for MBP-87–99 typically express the BV6 gene and are restricted by the RT-1B I-E molecule.[16–17]

During the recovery phase of EAE induced by sentitization with Gp-MBP/CFA, T cells appear that are specific for a third determinant, residues 55–69.[10] Pre-immunization with Gp-MBP-55–69 induced protection against active EAE.[11] As a T cell epitope, the Gp-MBP-55–69 peptide is not cross-reactive with Rt-MBP-55–69, differing by an histidine-alanine (HA) insertion at positions 61 and 62, and by an alanine for threonine substitution at position 63 (Table 1). In fact, the Rt-MBP-55–69 peptide was relatively non-immunogenic in the Lewis rat.

In a previous report, we isolated a T cell clone specific for Gp-MBP-55–69 that expressed the BV8S6 gene, and was restricted by the RT-1B I-A molecule. This T cell clone was not encephalitogenic in Lewis rats, but instead induced protection against both active and passive EAE directed against the Gp-MBP-72–89 determinant.[10–11] The BV8S2 and BV8S6 proteins are highly homologous, containing a consensus sequence from residues 44–54 that is found within the CDR2. This region of the BV8S2 molecule has been of great interest to us because of the ability of the BV8S2-39–59 peptide to induce protection against EAE. The protective mechanism induced by the BV8S2-39–59 peptide involved regulatory T cells that could recognize and down-regulate MBP-specific T cells that expressed this BV8S2 gene.[3]

Protection induced by the Gp-MBP-55–69-specific T cell clone potentially could involve stimulation of the anti-TCR regulatory network through cross-reactivity between BV8S2 and BV8S6. This notion was supported by our subsequent studies in which T cell responses to the BV8S2-39–59 peptide could be induced after injection of the BV8S6$^+$ T cell clone specific for Gp-MBP-55–69, and in which protection against EAE could be induced by the BV8-44–54 consensus peptide.[11] Interestingly, the BV8S2-39–59 peptide effective against encephalitogenic BV8S2$^+$ T cells specific for Gp-MBP-72–89, was also cross-reactive with the BV6-39–59 peptide that was effective against encephalitogenic BV6$^+$ T cells specific for MBP-87–99.[16–17] Thus, the BV8S2-39–59 peptide was central in stimulating regulatory T cells that could inhibit responses to either encephalitogenic determinant. Of additional importance, T cell responses to the BV8S2 peptides were largely MHC I restricted,[18] suggesting recognition by the regulatory T cells of an MHC I bound peptide on the surface of the encephalitogenic MBP-specific T cells.

Recently, we characterized both rat and mouse MHC class I bound peptides eluted from rat-mouse hybridomas obtained by fusing Gp-MBP-72–89-specific T cells with the mouse BW-5147 fusion partner.[19–20] From these eluted peptides, we identified two distinct nonamer peptide motifs, including XXLXXXXXS (pattern 1) and XXXXXXXXL (pattern 2), thought to represent general patterns found in peptides that preferentially bind to two different RT-1B MHC class I molecules.[20] Interestingly, the BV8S2-42–50 peptide, HGLRLIHYS, contained MHC binding anchor residues identical to those found in pattern 1. Moreover, three BV8S2 peptides from the 71–95 region were identified that contained anchor residues similar to those found in pattern 2. This region of BV8S2 has also been of great interest to us, as injection of the 71–90 peptide can induce regulatory T cells that inhibited EAE induced with Gp-MBP-72–89/CFA.[21]

The biological activity of BV8S2 peptides containing MHC class I binding motifs is currently under careful investigation in Lewis rats. We anticipate that these peptides may effectively trigger MHC class I-restricted CD8$^+$ T cells that could target and eliminate encephalitogenic BV8S2$^+$ T cells naturally expressing these sequences. Alternatively, these or similar but slightly longer peptides might activate CD4$^+$ regulatory T cells that inhibit the encephalitogenic T cells through soluble suppressive factors, as we have shown previously, or promote differentiation of CD8$^+$ regulatory T cells. In either case, these peptides are likely to be involved in the TCR regulatory network.

Table 1 Proposed cross-reactive TCR and myelin basic protein sequences

Rt-MBP-58–74	*DSH*	*--IATTHYG*	*SLPQK*
Gp-MBP-58–74	*DSH*	*HAARTTHYG*	*SLPQK*
Rt-BV8S2-39–55	*DMG*	*HGLRLIHYS*	*YDVNS*
Rt-BV8S6-39–55	NMG	*DELRLIHYS*	*YDVNR*
Hu-MBP-58–74	*DSH*	*HPARTAHYG*	*SLPQK*
Hu-BV12S2-39–55	*DLG*	*HGLRLIHYS*	*YGVQD*
Hu-BV12S1-39–55	*DPG*	*HGLRLIHYS*	*YGVKD*

Identical residues indicated by bold and italics. Conservative substitutions indicated by italics only. Underlined residues indicate proposed anchor positions for binding to RT-1B MHC class I molecules.

Sequence homology between BV8S2-39–59 and Gp-MBP-55–69

Having identified possible MHC class I binding motifs and their associated anchor residues, we evaluated the Gp-MBP-55–69 peptide for homology with the BV8S2 regulatory sequences and for the presence of either of these motifs. As is shown in Table 1, the Gp-MBP-61–69 and BV8S2-42–50 nonamer peptides had four identical residues (H61, R64, H67 and Y68) and other potentially permissive substitutions, with additional residues on either side of this region being identical or compatible. This similarity between the two peptides suggested that inhibition of EAE induced by injecting the Gp-MBP-55–69 peptide, in addition to inducing T cells expressing cross-reactive V genes, might also involve direct cross-reactivity with the regulatory BV8S2-39–59 peptide.

Immunological cross-reactivity between BV8S2-39–59 and Gp-MBP-55–69

To evaluate possible cross-recognition between rat BV8S2-39–59 and Gp-MBP-55–69 sequences, we immunized Lewis

rats with either peptide in adjuvants and tested for cross-reactive T cells and antibodies. As is shown in Fig. 1, lymph node (LN) cells and T cell lines from rats immunized with BV8S2-39–59 peptide had strong proliferation responses to the immunizing peptide, and substantial cross-reactivity to the Gp-MBP-55–69 peptide. This strong proliferation response was retained when the T cells were stimulated with the BV8S2-42–52 12-mer peptide, but almost all proliferation response was lost when the T cells were stimulated with the BV8S2-2–50 nonamer peptide (data not shown). Conversely, LN cells and T cell lines from rats immunized with Gp-MBP-55–69 peptide proliferated vigorously in the presence of the immunizing peptide, but failed to respond to the BV8S2-39–59 peptide. However, this pattern showing a one-way cross-reactivity to Gp-MBP-55–69 in rats immunized with rat BV8S2-39–59 peptide was not reflected by delayed-type hypersensitivity (DTH) responses (Fig. 2) or by serum antibody responses (Fig. 3). In both cases, vigorous responses were observed to the immunizing peptides, but not to the other non-immunizing peptides.

Implications of cross-reactivity

These data demonstrate that responses induced with the rat BV8S2-39–59 peptide can induce helper T cell responses but not antibody recognition of a cross-reactive determinant represented within the Gp-MBP-55–69 sequence. However, this cross-reactivity was unidirectional, as T cells responsive to Gp-MBP-55–69 did not recognize the rat BV8S2-39–59 peptide. The MHC class I-associated nonamer peptides were apparently too short to induce proliferative responses in T cells stimulated initially with longer peptides, but their recognition in association with MHC I molecules by cytotoxic or regulatory T cells is still under study as of this

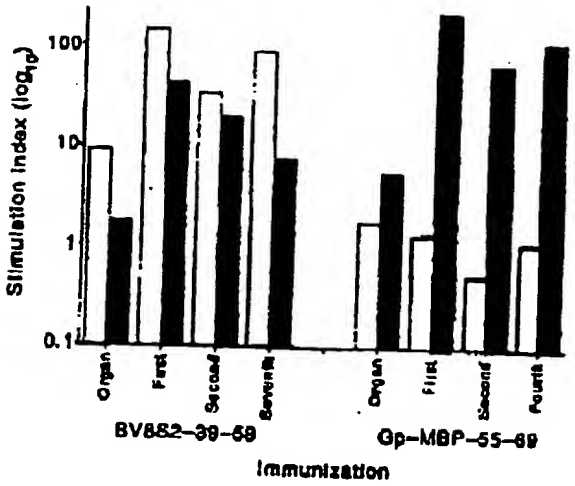

Figure 1 T cell cross-recognition of (■) Gp-MBP (GPBP)-55–69 peptide after immunization with rat (□) BV8S2-39–59 peptide. Lewis rats were immunized with either peptide in CFA and, after 28 days, lymph node cells were removed and cultured with each peptide at several dilutions (organ cultures). Some cells from each preparation were selected into T cell lines by cyclically expanding the cells in IL-2 and then restimulating with peptide presented by irradiated thymocytes as antigen-presenting cells. Responses, given as the stimulation index (c.p.m. with peptide/c.p.m. without peptide), are presented for various stimulations of the T cell lines. Note strong proliferation response to Gp-MBP-55–69 by T cell line specific for BV8S2-39–59, but not the reverse.

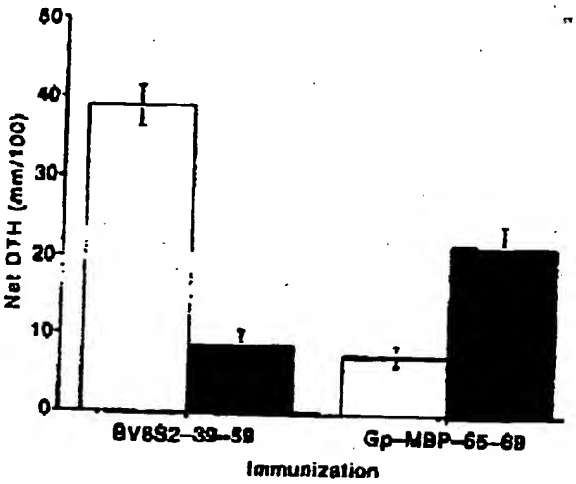

Figure 2 Weak cross-reactivity between (□) BV8S2-39–59 and (■) Gp-MBP-55–69 as assessed by delayed-type hypersensitivity (DTH) reactions. Fourteen days after immunization with either peptide in CFA, Lewis rats were tested for DTH responses by injecting 20 μg of each peptide intradermally in the ear pinna. Differences in ear thickness were measured by a pressure-sensitive micrometer before injection, and 24 and 48 h after injection. Data represent the 24 h reading. Note strong DTH reactions against the immunizing peptide, and weak responses to the other peptide.

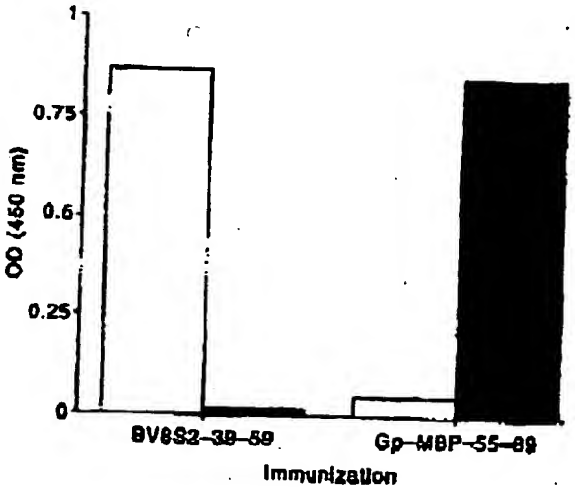

Figure 3 Lack of cross-reactivity between (□) BV8S2-39–59 and (■) Gp-MBP-55–69 as assessed by serum antibody reactivity. Serum was collected 28 days after immunization of Lewis rats with either peptide in adjuvant. The serum was tested at various dilutions by ELISA for reactivity to the immunizing and non-immunizing peptides. Data are from the 1/100 serum dilution. Note strong reactivity to the immunizing peptide, but a lack of activity to the non-immunizing peptide.

writing. One important issue remaining to be resolved is whether the two nonamer sequences are also cross-reactive, in either a uni- or bidirectional manner.

The lack of T cell recognition of the BV8S2-39–59 determinant after immunization with Gp-MBP-55–69 *does not support* the idea that natural sensitization with MBP can directly induce regulatory TCR-specific T cells. Firstly, Rt-MBP lacks this T cell determinant, and could not be expected to amplify T cell responses *in vivo*. However, the one-way cross-reactivity described above may still play an important role in the induction of both MHC class I- and class II-restricted T cells specific for TCR determinants. As proposed earlier,[11] the protective effect of Gp-MBP-55–69 would appear to be indirect, through induction of non-encephalitogenic T cells that express V genes that cross-react with regulatory TCR sequences. These T cell-associated V region determinants may well be more immunogenic than soluble TCR peptides, especially for inducing MHC class I-restricted responses. Thus, vaccination with soluble TCR peptides, in addition to directly activating MHC class II-restricted TCR-specific T cells after uptake and processing by tissue-specific accessory cells, may expand the circulating population of non-encephalitogenic MBP-55–69-specific T cells. These T cells could then act as a cellular stimulus for regulatory TCR-specific T cells responding to MHC class I or II associated determinants.

Response to TCR peptide vaccination can inhibit T cell responses to MBP and may reverse clinical progression in some patients with MS

We carried out a pilot double-blind, placebo-controlled Phase II trial to evaluate the ability of the native and Y49T substituted BV5S2-38–58 peptides to induce TCR-specific T cells, inhibit MBP-specific T cells, and effect clinical changes.[22] The results of this trial indicated that (i) only patients who were vaccinated with peptides had significant increases in T cell response to peptides (as assessed by frequency of PBMC); (ii) a significant inverse correlation between response to peptide and response to MBP; (iii) a significant correlation between response to peptide and lack of disease progression; and (iv) a significant inverse correlation between response to MBP and lack of clinical progression. These data suggest that TCR peptide vaccination may affect the course of MS in some patients by inducing peptide-specific T cells.

Degree of response to BV5S2-38–58 is correlated with clinical benefit

To better view the collective effects of TCR peptide vaccination directed at BV5S2$^-$ T cells, we assembled a composite analysis of 32 DR2$^+$ patients with progressive MS from both our Phase I[23] and Phase II[22] trials, comparing the degree of response to the substituted or native BV5S2-38–58 peptide with clinical outcome.[24] We found a highly significant correlation ($P < 0.001$) between the degree of response to the BV5S2 peptide and clinical benefit. That there was measurable clinical improvement in three of the patients with strong T cell responses to peptide vaccination (≥ 8 cells/million), and arrested disease progression in seven of nine patients with moderate T cell responses (2–8 cells/million), underscores the exciting clinical potential of this approach, even in this small group of patients with rather advanced disease.

Mechanism of inhibition involves Th2 regulatory cells and bystander suppression

In order to develop a rational strategy for optimizing the TCR peptide vaccine, it is important to consider the biological characteristics of BV5S2-38–58-specific T cells, as well as their mode of action for regulating neuroantigen-specific Th1 cells that are thought to contribute to the disease process. To date, we have isolated and characterized more than 80 clones responsive to BV5S2-38–58, BV6S1-38–58, or BV9-39–59.[22,25,26] The vast majority of these clones were CD4$^+$, and most (about 75%) produced Th2 lymphokines, including IL-4, IL-5, and IL-10, but not Th1 lymphokines such as IFN-γ, IL-2, or TNF. The remainder produced both Th2 and Th1 lymphokines. Supernatants from these TCR peptide-reactive cultures had potent inhibitory properties when incubated with autologous or heterologous Th1 clones specific for MBP or Herpes simplex virus. The supernatants inhibited both antigen-specific and mitogen-induced proliferation, as well as IFN-γ release, in a dose-dependent, but TCR V gene-independent manner.[26] Careful titration of IL-10 in the supernatants revealed that the detected levels (1.1 – 0.7 ng/mL) could account for inhibition produced by similar levels of recombinant human IL-10, and the actual involvement of IL-10 was demonstrated by neutralizing the inhibitory properties of the supernatant with anti-IL-10 antibody.[27] No inhibition of Th1 cells was observed when supernatants from Th1 cultures were added, or when free TCR peptides were added. Conversely, no inhibition of Th2 cells was noted when supernatants from Th2 cells (that inhibited Th1 cells) were added. Thus, regional activation of the TCR peptide-specific T cells (e.g. by Th1 cells expressing BV5S2, or APC presenting soluble substituted or native BV5S2-38–58 peptide after vaccination) would be expected to induce relatively high concentrations of soluble IL-10 that would inhibit activation of any Th1 cells, including those that expressed V genes different than the target T cell (e.g. BV5S2$^+$) in the nearby vicinity.

Hu-MBP-55–74 is a human T cell determinant

Similar to the immunogenic Gp-MBP-55–69 sequence and distinct from the non-immunogenic Rt-MBP-55–69 peptide, the Hu-MBP-55–74 sequence also contains an insertion at positions 61 and 62 (Table 1). We have evaluated MBP-specific T cells from both MS patients and controls, and have consistently found responses to the Hu-MBP-55–74 peptide, as is shown in Fig. 4 for two Hu-MBP-55–74-specific T cell clones from a normal donor. Although it is unknown if Hu-MBP-55–74 is encephalitogenic in humans, the consistent appearance of T cells specific for this determinant in non-diseased individuals would argue against this possibility.

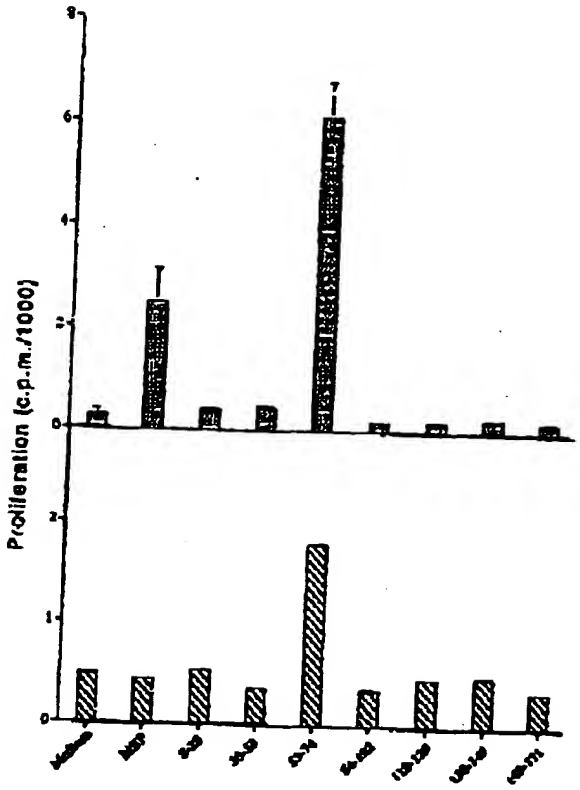

Figure 4 Proliferation responses of two T cell clones, (▲) clone 1 and (■) clone 2, specific for Hu-MBP-55–74 from a normal donor (RJ). Peripheral blood mononuclear cells (PBMC) at limiting dilution were stimulated with Hu-MBP, expanded in IL-2, and then repeatedly restimulated with Hu-MBP presented by irradiated PBMC to make T cell clones. After six rounds of selection, the T cell clones were stimulated with the indicated peptides. Note the selective proliferation response to the Hu-MBP-55–74 peptide.

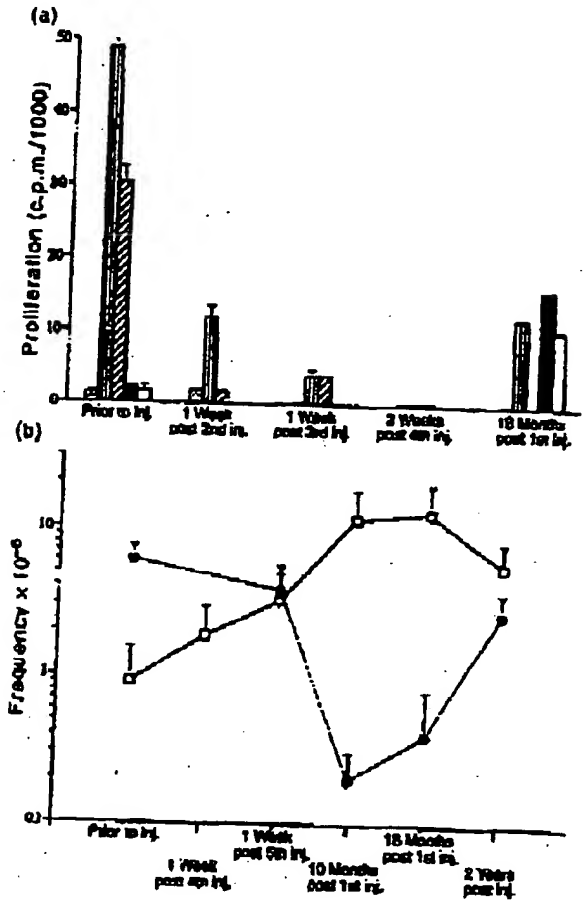

Figure 5 Evaluation of T cell responses to myelin basic protein (MBP) and TCR BV12S2-38–58 peptide in multiple sclerosis patient FA before and during vaccination with the BV12 peptide. (a) The patient was injected with 100–200 μg BV12S2 i.d. in saline once weekly (4×), and then monthly for 2 years. Proliferation responses to the indicated human (Hu)-MBP peptides are shown for T cell lines selected before and during vaccination. The cell lines were: (▨) medium; ▩ myelin basic protein; (▩) 84–102; (■) 55–74; and (□) 149–171. Note initial response to Hu-MBP-84–102 peptide, which switched during TCR peptide therapy to Hu-MBP-55–74 and -149–171 peptides. (b) The frequencies of T cells specific for (●) Hu-MBP and (□) BV12S2-38–58 were estimated by limiting dilution during TCR peptide vaccination. Note strong initial response to Hu-MBP, which declined as the patient developed a strong response to vaccination.

Epitope switching to Hu-MBP-55–74 in BV5S2-38–58 peptide-vaccinated MS patients

Interestingly, successful vaccination with BV5S2-38–58 peptide not only decreased the frequency of MBP-specific T cells,[22] but also induced a shift in the pattern of epitopes recognized by an MS patient. As is shown in Table 2, patient RM had only one of six MBP-specific T cell clones that responded selectively to the MBP-55–74 peptide prior to TCR peptide vaccination, with the remaining clones responsive to peptides 8–28, 87–99, and 90–170. This clone expressed TCR BV6, which we subsequently showed contained a CDR2 determinant cross-reactive with the immunizing BV5S2-38–58 peptide. After 5 years of vaccination with BV5S2-38–58 peptide, resulting in clinical stabilization, MBP-specific T cell clones from patient RM were again isolated and tested for specificity and, in some cases, BV gene expression. Surprisingly, five of 10 clones were specific for MBP-55–74, with four clones specific for the 35–55 peptide and only one clone specific for the immunodominant 84–102 peptide. Again, one clone specific for MBP-55–74 expressed TCR BV6 (the only one analysed), consistent with the idea that T cells specific for the MBP epitope may express BV genes that cross-react with putative pathogenic specificities.

Peptide sequence homology between Hu-MBP-55–69 and BV12

The striking sequence homology shown above between Gp-MBP-61–69 and rat TCR BV8S2-42–50 suggested that a similar homology might be present between the corresponding segments of Hu-MBP and TCR BV12, the human

Table 2 Change of epitope specificity and TCR usage of myelin basic protein (MBP)-reactive T cells from multiple sclerosis patient RM (DR2 homozygous)

	Before TCR immunization* (1989–1990)						
		Clone					
Ag	Line	#21	#22	#41	#43	#48	#51
MBP	26.3	5	0.7	3.7	1.3	2	3.2
8–28	3.3	0	0	0	2	0	0
55–74	12.1	0	0	0	0	0	1.8
87–99	17.1	1.5	0	1.2	0	0	0
90–170	8	0	0.5	0	0	1.1	0
(TCR usage)	ND	Vβ5.2	Vβ5.2	Vβ5.2	Vβ5.2	Vβ6.1	Vβ6.1

	After immunization with TCR peptides for 5 years* (1996)									
	Line									
Ag	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10
MBP	0	1.4	2.9	45	27	7	4	22	3	17
8–28	0.1	0	0.2	0.4	0	ND	ND	1	1	0
35–55	0	0.1	0.9	32	18	0	0	75	16	0
55–74	41	7.9	18	0	0	7	8	0	0	0
84–102	0.1	0.1	0	0	0	0	0	0	0	18
149–171	0	0	0.2	0.4	0	ND	ND	0	0	ND
(TCR usage)	Vβ6	ND	ND	Vβ20	Vβ20	ND	ND	ND	ND	ND

*Proliferation expressed as net c.p.m. (1×10^3) by subtracting background c.p.m. $\leq 0.5 \times 10^3$ from experimental c.p.m.

homologue for the rat BV8 gene. This was indeed the case, with the same four identical residues being present within the Hu-MBP-61–69 and Hu-BV12S1/BV12S2-42–50 nonamer sequences (Table 1). In addition, there was identity at D58 and Q73 adjacent to the nonamer, with amino acids at other positions being potentially permissive.

Epitope switching to Hu-MBP-55–74 in BV12S2-38–58 peptide-vaccinated MS patients

The cross-reactivity between Rt-BV8S2-39–59 peptide and Gp-MBP-55–69 raised the intriguing possibility that vaccination with Hu-BV12 CDR2 peptide might also stimulate T cell responses to Hu-MBP-55–74. Although most of the MS patients in our TCR peptide therapy programme have been vaccinated with BV5 peptides (based on over-expression of BV5 by MBP-specific T cells), one MS patient (FA) was treated with BV12S2-38–58 peptide. During the course of treatment over a 2 year period with this peptide, the patient was evaluated for both frequency and epitope specificity of MBP-reactive T cells. As is shown in Fig. 5, patient FA began therapy with a very high frequency of MBP-reactive T cells (8/million PBMC), with predominant T cell reactivity to the immunodominant Hu-MBP-84–102 peptide and essentially no response to a second dominant epitope, MBP-149–171, or to MBP-55–74. After injection of BV12S2 peptides, the frequency of MBP-specific T cells dropped to < 1 cell/million, and responses disappeared to all of the MBP epitopes tested, concomitant with a striking increase in response to the vaccinating BV12S2 peptide (to > 15 cells/million) and clinical stabilization. Interestingly, after 18 months of treatment, response to MBP began to reappear. However, this response was no longer directed against the immunodominant MBP-84–102 determinant that we have associated with clinical episodes in some patients with relapsing MS. Instead, the response had switched to the 55–74 and 149–171 determinants!

In accordance with our previously published clinical trials using BV5S2 peptides, these data suggest that induction of T cell responses to TCR peptides can inhibit potentially pathogenic T cells specific for dominant epitopes of MBP. Moreover, the appearance of T cells specific for MBP-55–74 during therapy mimics precisely the pattern of epitope switching that we previously observed in Lewis rats treated with BV8S2 peptides.[6] Given the sequence homology, it is possible that this epitope switch to MBP-55–74 may have been stimulated, in part, by successful vaccination with a cross-reactive determinant present on the BV12S2 peptide. Experiments are currently in progress to test cross-recognition of Hu-MBP-55–74 by T cells specific for the BV12S2-38–58 peptide, and to determine if the MBP-55–74-specific T cells express BV genes with CDR2 sequences that are cross-reactive with BV12S2.

Conclusion

The immunoregulatory mechanisms induced by vaccination with TCR peptides include the induction of peptide-specific T cells and antibodies that can interact with the target pathogenic T cells, thereby inhibiting response to neuroantigen. During TCR peptide vaccination in both rats with EAE and humans with MS, we have observed striking changes in the specificity of these neuroantigen-specific T cells that supports the idea that the immunoregulation causes a switch from pathogenic to benign specificities. In the rat model of EAE, the Gp-MBP-55–69 determinant is not only benign, but also protective against EAE. This protection involves the induction of MBP-55–69-specific T

cells expressing cross-reactive V genes that, as cell associated antigens, may be more effective immunogens than soluble TCR peptides. In this review, we have presented new data suggesting immunological cross-reactivity between MBP and TCR V gene sequences that could result in the expansion of MBP-55–69-reactive T cells, thereby providing additional cell-associated TCR determinants that might effectively induce stronger regulatory immunity, particularly $CD8^-$ T cells that may best recognize TCR determinants in the context of cell-associated MHC class I molecules. We thus propose that the MBP-55–69 determinant represents a protective rather than a pathogenic epitope.

The data presented above from two MS patients treated long-term with TCR peptides are consistent with our observations in rats with EAE. That is, in the first example, MBP-55–74-specific T cells that appeared in patient FA after vaccination with the BV12S2-38–58 peptide could have been induced by direct cross-reactivity, similar to the ability of the rat BV8S2-39–59 peptide to induce T cell responses to MBP-55–69. Alternatively, cross-reactive BV genes could have been involved, although we present no data to support this idea. In the second example, there was an apparent skewing in the percentage of MBP-specific T cell clones towards the MBP-55–74 specificity, with expression of BV genes known to cross-react with the vaccinating TCR BV8S2 sequence, again similar to epitope switching to MBP-55–74 in TCR peptide-vaccinated rats. These data suggest that either or both mechanisms involving the MBP-55–74 peptide itself, or T cells specific for the MBP-55–74 peptide may activate potentially protective TCR peptide T cells present in MS patients.

Acknowledgements

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Immunology

A T cell receptor antagonist peptide induces T cells that mediate bystander suppression and prevent autoimmune encephalomyelitis induced with multiple myelin antigens

(altered peptides/immune deviation/regulatory T cells)

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ABSTRACT Experimental autoimmune encephalomyelitis (EAE) induced with myelin proteolipid protein (PLP) residues 139–151 (HSLGKWLGHPDKF) can be prevented by treatment with a T cell receptor (TCR) antagonist peptide (L144/R147) generated by substituting at the two principal TCR contact residues in the encephalitogenic peptide. The TCR antagonist peptide blocks activation of encephalitogenic Th1 helper cells *in vitro*, but the mechanisms by which the antagonist peptide blocks EAE *in vivo* are not clear. Immunization with L144/R147 did not inhibit generation of PLP-(139–151)-specific T cells *in vivo*. Furthermore, preimmunization with L144/R147 protected mice from EAE induced with the encephalitogenic peptides PLP-(178–191) and myelin oligodendrocyte protein (MOG) residues 92–106 and with mouse myelin basic protein (MBP). These data suggest that the L144/R147 peptide does not act as an antagonist *in vivo* but mediates bystander suppression, probably by the generation of regulatory T cells. To confirm this we generated T cell lines and clones from animals immunized with PLP-(139–151) plus L144/R147. T cells specific for L144/R147 peptide were crossreactive with the native PLP-(139–151) peptide, produced Th2/Th0 cytokines, and suppressed EAE upon adoptive transfer. These studies demonstrate that TCR antagonist peptides may have multiple biological effects *in vivo*. One of the principal mechanisms by which these peptides inhibit autoimmunity is by the induction of regulatory T cells, leading to bystander suppression of EAE. These results have important implications for the treatment of autoimmune diseases where there are autopathogenic responses to multiple antigens in the target organ.

T cell epitopes derived from protein antigens may induce immunity or suppression (1). An immunogenic epitope can be converted to a suppressor epitope by changing a single amino acid. A clear example of this was described in H-2^s mice which develop helper T cell responses to the copolymer of Glu and Ala (GA) and tolerogenic responses to the copolymer of Glu, Ala, and Tyr (GAT) (2, 3). Therefore substitution of tyrosine in the GA polymer induced suppressor T cells that were crossreactive with the immunogenic GA molecule and converted immunogenic GA into an immunosuppressive molecule. This result was attributed to distinct binding of immunogenic and tolerogenic epitopes to different H-2-linked genes (*Ir* versus *Is* genes). However, the failure to identify distinct *Is* genes has led researchers in the field to reexamine this phenomenon. We have been studying the mechanisms by which autoimmune disease can be regulated with a view that the nature and role of regulatory cells can be tested *in vivo* in an autoimmune disease setting.

Analogs of encephalitogenic peptides have been known to protect animals from the induction of experimental autoimmune encephalomyelitis (EAE) for a number of years (4, 5). Originally it was postulated that the principal mechanism by which peptide analogs mediate protection was major histocompatibility complex (MHC) blockade, and this was shown to be the case for peptides with high affinity for MHC class II molecules (6, 7). However, a number of observations raised the possibility that MHC blockade was not the only mechanism responsible for protection (8, 9). The description of altered peptide ligands generated by single amino acid substitution of the antigenic peptide that could act as T cell receptor (TCR) antagonists or partial agonists of various T cell clones provided a framework to understand the actions of many peptide analogs (10–13). We have shown that an analog of the encephalitogenic myelin proteolipid protein (PLP)-(139–151) peptide (14–16), L144/R147 (with substitutions at the two main TCR contact residues), is a powerful TCR antagonist for encephalitogenic PLP-(139–151)-specific T cell clones *in vitro* and is able to protect animals from the induction of EAE (17). This effect was in contrast with that of a second, weaker, TCR antagonist analog L144, which had little or no effect on the development of clinical disease. While the *in vivo* protective effects of L144/R147 could have been due to antagonism of PLP-(139–151)-specific T cells, the observation that mice coimmunized with L144/R147 plus the encephalitogenic peptide PLP-(139–151) and without signs of clinical disease developed inflammatory foci within the central nervous system suggested that L144/R147 was not simply ablating EAE by inhibiting the development of a PLP-(139–151)-specific T cell response.

To investigate the mechanism by which the TCR antagonist peptide L144/R147 mediated its protective effects *in vivo*, we tested the effects of L144/R147 on the induction of EAE with other unrelated encephalitogenic peptides and proteins derived from central nervous system myelin. We found that, as well as protecting from EAE induced with the encephalitogenic peptide PLP-(139–151), the L144/R147 peptide can protect animals from EAE induced by another encephalitogenic PLP peptide and by the unrelated myelin antigens myelin basic protein (MBP) (18) and myelin oligodendrocyte glycoprotein (MOG) (19), excluding the possibility that protection was simply due to TCR antagonism *in vivo*. Our data further suggest that the L144/R147 altered peptide induces bystander

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Abbreviations: MHC, major histocompatibility complex; TCR, T cell receptor; PLP, myelin proteolipid protein; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MOG, myelin oligodendrocyte protein; Nase, neuraminidase; CFA, complete Freund's adjuvant; LNC, lymph node cells; IFN, interferon; IL, interleukin; PCC, pigeon cytochrome *c*.

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suppression by generating regulatory T cells that crossreact with the native encephalitogenic peptide PLP-(139–151) and that transfer of these crossreactive regulatory T cell lines can protect against the development of EAE.

MATERIALS AND METHODS

Animals. Four- to 6-week-old female SJL mice were purchased from The Jackson Laboratory and housed under virus-free conditions. They were maintained in accordance with the guidelines of the Committee on Animals of Children's Hospital and Harvard Medical School.

Antigens. Peptide antigens were synthesized by Richard Laursen on a Milligen model 9050 synthesizer using Fmoc chemistry. Milligen PAL amide resins were used to produce peptides with C-terminal amides. Most peptides were >90% pure, as determined by HPLC. The peptides used in these experiments were PLP-(139–151) (HSLGKWLGHPDKF), L144 (HSLGKLLGHPDKF), Q144 (HSLGKQLGHPDKF), L144/R147 (HSLGKLLGRPDKF), PLP-(178–191) (NTWTTCQSIAFPSK), MOG-(92–106) (DEGGYTCFFRDHSYQ), and neuraminidase (Nase)-(101–120) (EALVRQGLAKVAYVYKPNNT). MOG-(92–106) and mouse MBP were the kind gift of A. Al-Sabbagh (Autoimmune Inc., Lexington, MA).

Preimmunization, Induction, and Assessment of EAE. Mice were preimmunized by injecting s.c. at two sites with the relevant peptide (100 or 200 μg per mouse) emulsified in complete Freund's adjuvant (CFA; Difco) supplemented with *Mycobacterium tuberculosis* H37 RA (500 μg per mouse; Difco). Three to 6 weeks later mice were immunized with the disease-inducing peptide or protein (25–200 μg) emulsified in CFA and supplemented with *M. tuberculosis* H37 RA (500 μg per mouse). On this day and on day 3 after immunization each mouse was also injected i.v. with 10^9 heat-killed *Bordetella pertussis* bacilli (pertussis vaccine lot no. 285, Massachusetts Public Health Biological Laboratories, Boston). Mice were examined daily, beginning on day 9, for disease, which was assessed clinically according to the following criteria: 0 = no disease, 1 = limp tail, 2 = hindlimb weakness, 3 = hindlimb paralysis, 4 = hindlimb plus forelimb paralysis, and 5 = moribund or dead.

***In Vitro* Proliferation and Cytokine Assays.** Mice were injected s.c. at five sites with antigen emulsified in CFA containing a total of 250 μg of *M. tuberculosis* H37 RA. Mice immunized with a single peptide received a total of 100 μg of antigen; mice immunized with a mixture of PLP-(139–151) and an analog peptide received 100 μg of PLP-(139–151) and either 100 μg or 300 μg of second peptide (i.e., a total of 200 or 400 μg of antigen per mouse). On day 12 lymph nodes were removed and lymph node cells (LNC) were prepared from them. LNC (4×10^5 per well) were cultured in triplicate in 96-well round-bottom plates (Falcon, Becton Dickinson), in the presence of antigen, for 48 hr, then [^{3}H]thymidine [1 μCi (37 kBq) per well] was added for the last 16 hr before harvesting the cells. [^{3}H]Thymidine incorporation was determined in triplicate wells in a Beckman scintillation counter (model LS 5000). The data were expressed as a stimulation index, which was calculated by dividing the proliferation (cpm incorporated) measured in the presence of antigen by the proliferation measured with medium alone.

To measure the concentration of cytokines, supernatants were collected from activated T cells (5×10^4 T cells plus 5×10^5 syngeneic irradiated spleen cells per well in the presence or absence of antigen), 40 hr after activation and diluted with an equal volume of fresh culture medium. Interferon (IFN)-γ and interleukins IL-2, IL-4, and IL-10 were measured by quantitative capture ELISA according to the supplier's guidelines as previously described (20).

Derivation and Adoptive Transfer of T Cell Lines and Clones. T cell lines were generated from LNC from mice immunized with PLP-(139–151) plus L144/R147 and cultured in syngeneic serum with the appropriate antigen (20 μg/ml) for 5 days. T cell blasts were purified over a Ficoll/Hypaque gradient and expanded. Long-term culture and cloning were carried out as described (20).

T cell lines for adoptive transfer were prepared from mice immunized by using the same protocol used for the *in vitro* proliferation assays. Lymph nodes were removed on day 10 and LNC were resuspended at a concentration of $6–10 \times 10^6$ cells per ml in culture medium containing 0.5% syngeneic serum in place of fetal bovine serum. Cells were cultured in the presence of various antigens (20 μg/ml) for 4 days, then harvested and purified over a Ficoll/Hypaque gradient. Cells were resuspended in PBS at 25×10^6 cells per ml and injected i.v. into recipient animals (0.2 ml, 5×10^6 cells per mouse), then recipient mice were immunized with the peptide PLP-(178–191) (10–100 μg per mouse) as described above to induce active EAE.

RESULTS AND DISCUSSION

We generated a peptide analog of the encephalitogenic peptide PLP-(139–151) by replacing the two principal TCR contact residues within the peptide with leucine (L) at position 144 and arginine (R) at position 147. This peptide analog (L144/R147) acted as a TCR antagonist for encephalitogenic Th1 clones, blocking their activation *in vitro*. Furthermore, coimmunization of SJL mice with a mixture of the encephalitogenic PLP-(139–151) peptide and the TCR antagonist peptide L144/R147 resulted in inhibition of disease (17). It was assumed that the mechanism by which L144/R147 peptide inhibited autoimmune disease depended upon antagonism preventing the generation of encephalitogenic T cells *in vivo*. However, the observation that L144/R147 plus PLP-(139–151) coimmunized mice that are protected from developing EAE have T cells which invade the central nervous system suggested that L144/R147 may inhibit EAE by mechanisms other than simply antagonizing the induction and activation of PLP-(139–151)-specific T cells. Two experiments were performed to determine whether L144/R147 inhibited EAE only by acting as a TCR antagonist *in vivo*. The L144/R147 peptide was tested for its ability to inhibit the induction of PLP-(139–151)-specific T cells and to prevent EAE induced *in vivo* with other myelin antigens.

To determine if the L144/R147 antagonist peptide could block the generation of PLP-(139–151)-specific T cells, mice were coimmunized with L144/R147 and PLP-(139–151). L144 peptide, which is also a TCR antagonist, was used as a control in these experiments. LNC were derived from animals immunized with PLP-(139–151), PLP-(139–151) plus L144, and PLP-(139–151) plus L144/R147 (Fig. 1 *a–c*). The proliferative response of LNC to PLP-(139–151) was not significantly reduced by coimmunization with either of the antagonists. On the other hand, immunization with L144/R147 alone induced T cells which were activated by L144/R147 and could also respond to Q144 and PLP-(139–151) at high concentrations (Fig. 1*d*). Therefore L144/R147 does not prevent the induction of PLP-(139–151)-specific cells, nor does it mediate MHC blockade *in vivo*, but it induces T cells that are crossreactive with the PLP-(139–151) peptide.

It was of interest that following immunization with a mixture of PLP-(139–151) with either the L144 or L144/R147 peptide, proliferative responses *in vivo* to L144 or L144/R147 alone were much lower than responses to PLP-(139–151) (Fig. 1 *b* and *c*). However, T cells specific for L144/R147 were generated in this coimmunization protocol, since we could derive L144/R147-specific T cell clones from mice immunized with PLP-(139–151) plus L144/R147 which responded specifically to L144/R147 (data not shown). A number of factors may contribute to the low responses to L144/R147 and L144 when

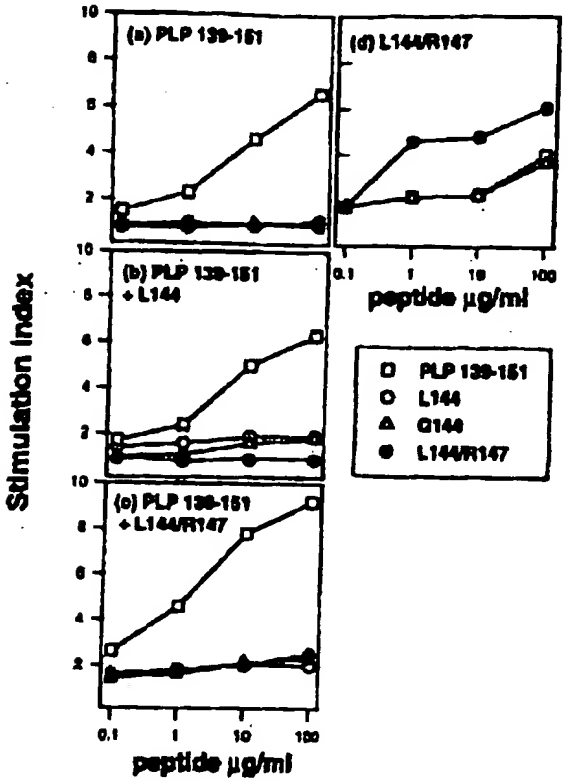

FIG. 1. The L144/R147 TCR antagonist does not block the generation of PLP-(139–151)-specific T cells. Groups of mice were immunized with 100 μg of PLP-(139–151) (*a*), 100 μg of PLP-(139–151) plus 300 μg of L144 (*b*), 100 μg of PLP-(139–151) plus 300 μg of L144/R147 (*c*), or 100 μg of L144/R147 (*d*). Twelve days after immunization LNC were removed and activated *in vitro* with the PLP-(139–151) native or analog peptides shown or with a control peptide (data not shown). Proliferation was assessed by adding [*methyl*-^{3}H]thymidine after 48 hr and harvesting 18 hr later, and a stimulation index was calculated. The background proliferation (cpm) of LNCs without antigen was 8,550 (*a*), 11,000 (*b*), 4,900 (*c*), and 1,970 (*d*).

mice are immunized with these peptides together with PLP-(139–151). The frequency of T cells able to respond to PLP-(139–151) may be greater than the frequency of T cells able to respond to L144/R147. In addition, the native PLP-(139–151) peptide may act as an antagonist for the induction of L144/R147- or L144-specific cells which may reduce the proliferative response *in vitro*. This effect is not due to differential binding of PLP-(139–151) over L144/R147 or L144, since all three altered peptides bind with similar affinity to $I-A^s$ (17).

To investigate whether the L144/R147 antagonist peptide affected disease induced with other myelin antigens or whether its effects were restricted to disease induced with the related peptide PLP-(139–151), mice were preimmunized with L144/R147 and disease was induced with a number of unrelated encephalitogens (Fig. 2 and Table 1). First we utilized a second encephalitogenic region within the PLP molecule, PLP-(178–191), and we found that L144/R147 protected mice against the development of EAE induced with this unrelated PLP peptide (Fig. 2*a*). The peptide had only a slight effect on the disease incidence but had a significant effect on the disease severity (Table 1). Mice preimmunized with L144 or the non-pre-immunized control mice developed disease that was significantly more severe than mice preimmunized with L144/R147. Disease following preimmunization with the native PLP-(139–151) developed earlier and was less severe than that of the mice preimmunized with L144 or not preimmunized, although these differences were not statistically significant (Table 1 and Fig.

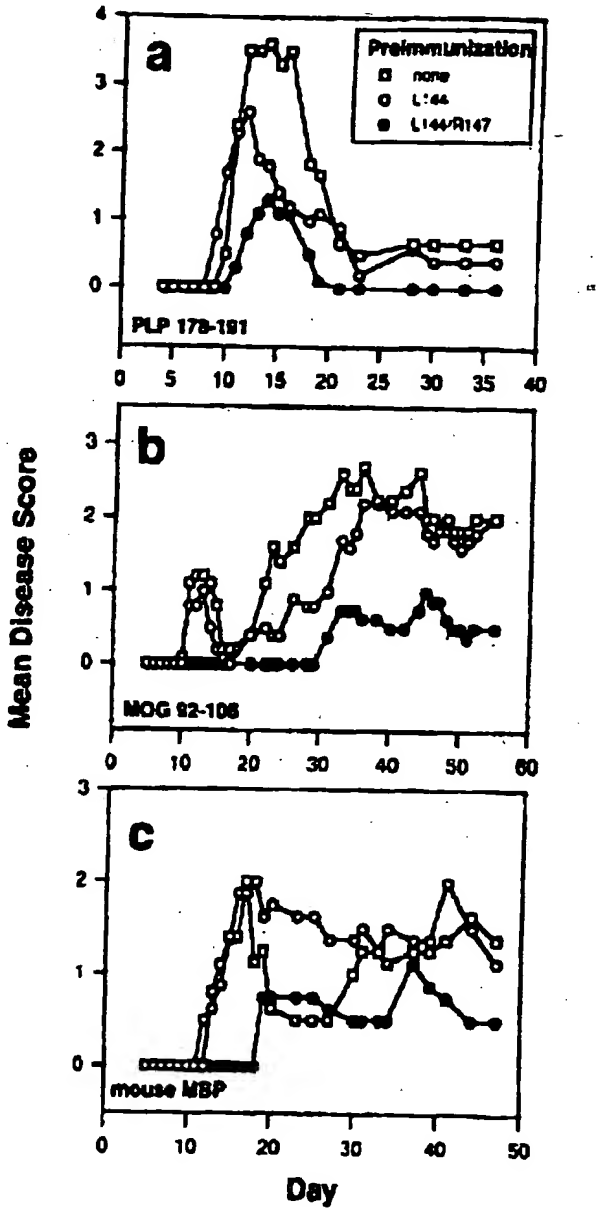

FIG. 2. Preimmunization with L144/R147 protects animals from EAE induced with PLP-(178–191) (*a*), MOG-(92–106) (*b*), or mouse MBP (*c*). Mice (four or five per group, 100–200 μg of peptide per mouse) were preimmunized with L144, L144/R147, or PLP-(139–151) or were not immunized 3 weeks before immunization with PLP-(178–191) (25 μg per mouse), MOG-(92–106) (200 μg per mouse), or mouse MBP (200 μg per mouse, 2 times). Mice immunized with mouse MBP received two immunizations with antigen 1 week or 1 month apart. In the latter case the second immunization was taken as day 0.

2*a*). These data suggest that the protective effects of L144/R147 did not depend on immunization with PLP-(139–151) and were not restricted to disease induced by that peptide.

To define whether the L144/R147 peptide would confer protection from disease induced with unrelated myelin antigens, groups of animals were preimmunized with various peptides and challenged with either the encephalitogenic peptide MOG-(92–106) or intact MBP. Mice preimmunized with L144 or PLP-(139–151) or unimmunized controls experienced an initial episode of mild disease after immunization with MOG-(92–106) which remitted spontaneously by day 20. This remission was followed by a much more severe relapse and chronic disease which was maintained through day 55 (Fig.

Table 1. Preimmunization of mice with the antagonist peptide L144/R147 protects mice from the induction of EAE with unrelated encephalitogens

		Acute disease			All disease		
Preimmunization	Immunization	Incidence	Mean day of onset	Mean maximum severity	Incidence	Mean day of onset	Mean maximum severity
None	PLP-(178–191)				14/14	11.0 ± 0.2	4.1 ± 0.2
L144					12/15	12.3 ± 1.6	3.8 ± 0.3
PLP-(139–151)					4/5	9.3 ± 1.0[a]	3.1 ± 0.3
L144/R147					11/15	12.3 ± 0.4[a]	2.6 ± 0.3[b]
None	MOG-(92–106)[c]	9/11	13.9 ± 1.0	1.8 ± 0.3	10/11	14.9 ± 1.7	2.7 ± 0.5
L144		3/11	14.9 ± 1.3	1.5 ± 0.2	10/11	17.2 ± 2.1	2.0 ± 0.3
PLP-(139–151)		4/4	15 ± 1.7	1.5 ± 0.3	4/4	15 ± 1.7	2.0 ± 0.4
L144/R147		1/9[d]	20	1	4/9[d]	34.6 ± 5.8[a]	2.5 ± 0.5
None	MBP[f]	7/9	16.9 ± 1.3	2.6 ± 0.6	8/9	18.5 ± 2.0	2.3 ± 0.5
L144		7/9	16.3 ± 0.9	2.0 ± 0.3	7/9	16.2 ± 0.9	2.0 ± 0.3
L144/R147		1/9[e]	19	3	3/9[g]	34.3 ± 8.2[h]	1.8 ± 0.6

Mice were preimmunized with the indicated antigens (100–200 μg per mouse in CFA) and challenged with encephalitogenic peptides/proteins as described. Results are mean ± SEM (except for incidence).

[a] $P < 0.05$ by *t* test compared with no preimmunization.

[b] $P < 0.005$ by *t* test compared with no preimmunization and $P < 0.05$ compared with L144.

[c] Acute disease is taken as disease onset on or before day 20.

[d] $P < 0.01$ by Fisher's exact test compared with no preimmunization or preimmunization with PLP-(139–151). $P < 0.05$ compared with preimmunization with L144.

[e] $P < 0.05$ by Fisher's exact test compared with no preimmunization or preimmunization with L144.

[f] Acute disease is taken as disease onset on or before day 25.

[g] $P < 0.05$ by Fisher's exact test compared with no preimmunization.

[h] $P < 0.05$ by Mann–Whitney U test compared with no preimmunization or preimmunization with L144.

2*b*). In comparison to unimmunized mice, animals preimmunized with the control peptide L144 or the native PLP peptide 139–151 showed no changes in the disease incidence, severity, or day of onset [Table 1, MOG-(92–106) group]. By contrast, the L144/R147 preimmunized mice were almost completely protected against the initial acute episode of disease induced with MOG-(92–106) [$P < 0.001$ compared with no preimmunization or preimmunization with the native PLP-(139–151) peptide, $P < 0.05$ compared with preimmunization with L144; Table 1] and showed a significantly delayed onset of disease (Table 1). Mice immunized with MBP also developed EAE, but the disease was significantly delayed and occurred in significantly fewer animals pretreated with L144/R147 compared with no preimmunization or preimmunization with L144 or a control IA^s binding peptide Nase-(101–120) (Fig. 2*c* and Table 1).

If the L144/R147 peptide acted *in vivo* only as an antagonist of PLP-(139–151)-specific T cells, it should have inhibited EAE induced with this peptide but should not have affected disease induced with the other myelin antigens. Because the antagonist L144/R147 peptide did inhibit disease induced with other unrelated myelin antigens, we concluded that immunization with the L144/R147 peptide can initiate bystander suppression. Furthermore, coimmunization with PLP-(139–151) was not necessary for this to occur and therefore TCR antagonism was not the only mechanism by which the L144/R147 peptide mediated protection *in vivo*. We then considered two other possible mechanisms: L144/R147 may selectively antagonize the generation of encephalitogenic PLP-(139–151) T cells, allowing the development of PLP-(139–151)-specific nonpathogenic regulatory cells, or it may itself induce regulatory cells which are crossreactive with both L144/R147 and PLP-(139–151) and which suppress disease.

Previous studies have shown that immunization with a tolerogenic form of the antigen induces effector suppressor cells which, when activated either by the antigen or by anti-idiotype-bearing transducer suppressor cells, mediate bystander suppression of responses to other antigens present with the immunogen (21). Although effector suppressor cells are antigen specific, once activated they produce factors that nonspecifically suppress immune responses to other antigens in the vicinity (22). This suggests that the final molecule that suppresses immune responses is probably an antigen-nonspecific regulatory molecule (probably a suppressive cytokine). Bystander suppression also occurs after oral administration of antigens (oral tolerance) (23). In that system it is due to transforming growth factor β and Th2 cytokine-producing regulatory T cells which suppress EAE and other autoimmune diseases in a tissue-specific manner (24, 25). To test whether the L144/R147 peptide induces regulatory T cells which transfer protection, animals were immunized with L144/R147 or an irrelevant IA^s-binding peptide, Nase-(101–120) or pigeon cytochrome *c* residues 92–104 [PCC-(92–104)]. LNC were harvested, reactivated *in vitro* for 5 days, and then transferred into mice that were simultaneously immunized with PLP-(178–191) to induce disease. Before transfer the LNC from mice immunized with L144/R147 were reactivated *in vitro* with either L144/R147 or PLP-(139–151) peptide and the LNC from control immunized mice were reactivated with the appropriate peptide. As an additional control in these experiments we included groups of mice that were not transferred LNC. In two experiments 7/10 mice that did not receive cells developed disease. This occurred 1–2 days later than for mice that received cells, and it suggests that cell transfer itself can change the pattern of disease onset. The adoptive transfer of cells from mice immunized with L144/R147 protected mice from disease, and the protection was greatest in the group of mice given cells activated *in vitro* with L144/R147 (Fig. 3). Data from two independent experiments show that adoptive transfer of short term T cell lines activated with the antagonist L144/R147 peptide lowers the incidence of disease to 4/10 compared with the group of mice transferred with T cell lines activated with the control peptide (8/10). Therefore the incidence of disease in the mice transferred with L144/R147 peptide-specific T cell line was half of that seen in the mice transferred T cell lines specific for the control peptide, although this difference did not reach statistical significance. There is no difference in the mean maximum severity between

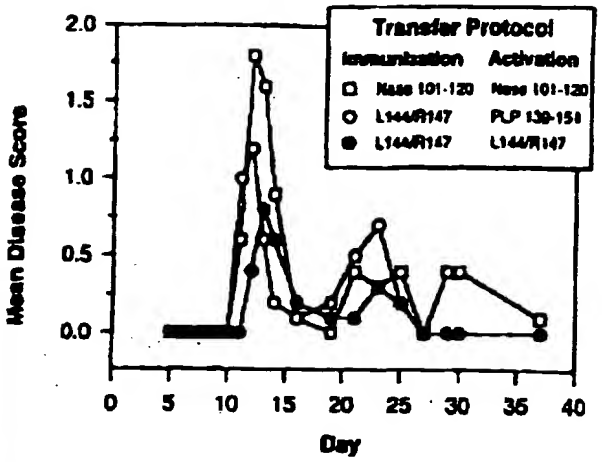

FIG. 3. Transfer of short term T cell lines from mice immunized with L144/R147 but not control peptide protects animals from disease. T cell lines were restimulated *in vitro* with L144/R147 or PLP-(139–151) (LNC from mice immunized with L144/R147) or with Nase-(101–120) or PCC-(92–104) [LNC from mice immunized with Nase-(101–120) or PCC-(92–104), respectively] and transferred i.v. (5×10^6 cells per mouse) at the time of immunization to induce active disease with a low dose of PLP-(178–191) (10–50 μg per mouse).

groups. It should be noted that in these experiments the T cell lines being tested for the protective effects are specific for the antagonist peptide of PLP-(139–151) and the mice are immunized with an unrelated encephalitogenic peptide PLP-(178–191) for the development of EAE. Thus transfer of antagonist peptide-specific T cells has to regulate the autopathogenic effects of encephalitogenic T cells that are continuously being generated and are specific for an unrelated encephalitogenic epitope. This demonstrates directly that L144/R147 peptide *in vivo* does not mediate its protective effects by antagonizing the generation of encephalitogenic T cells but induces regulatory cells that mediate the bystander suppression of EAE induced with a structurally unrelated encephalitogen.

To analyze the mechanism further, T cell lines and clones specific for the antagonist peptide L144/R147 were generated and tested for proliferative responses and cytokine production. Three independent lines were generated against the L144/R147 peptide. All of the T cell lines specific for the L144/R147 peptide generally produced cytokines of the Th0 phenotype, which included IFN-γ, IL-10, and IL-4 (data not shown). A panel of 16 T cell clones was established from one of the lines. Seven of these were crossreactive with PLP-(139–151) (data not shown). Eight stable clones that showed a significant antigen-specific response to L144/R147 were analyzed in Th0 phenotype. Five of the eight clones showed crossreactivity with the peptide PLP-(139–151) and four of the five clones that proliferated in response to this peptide also secreted detectable levels of the same cytokines (Table 2). None of the clones secreted significant levels of transforming growth factor β (data not shown). Analysis of TCR Vβ expression by five clones demonstrated a diverse repertoire with the expression Vβ6, Vβ14, and an unidentified TCR Vβ, showing that they were not sister clones (data not shown), although there was possibly an over-representation of Vβ6-bearing clones. Whereas the encephalitogenic PLP-(139–151) peptide induces Th1 (IFN-γ, tumor necrosis factor α) cells, our data demonstrate that immunization with the TCR antagonist peptide L144/R147 can induce crossreactive T cells which are of a Th2/Th0 phenotype and that lines derived from immunization with L144/R147 can regulate tissue destruction within the central nervous system and prevent clinical paralysis.

The altered peptide L144/R147 was originally designed as a TCR antagonist and was effective in blocking the activation of Th1 clones specific for PLP-(139–151) *in vitro*, in spite of the diversity of TCR αβ chains utilized by the clones. This broad inhibitory capacity could be rationalized on the basis of the finding that all T cell clones analyzed recognized the peptide/MHC in very similar ways and, crucially, all utilized the same primary TCR contact residue (W144). The L144/R147 peptide was also found to be an effective inhibitor of EAE *in vivo* when given either at the time of immunization or at the onset of clinical disease (17). However, the mechanisms by which a TCR antagonist peptide mediates protection against autopathogenic T cell responses *in vivo* have not been determined (17, 26, 27). Our results illustrate the complexity of the mechanisms underlying this phenomenon. Direct antagonism of PLP-(139–151)-specific T cells probably accounts for some of the ability of L144/R147 to ameliorate EAE when given at the onset of disease (17), but as the data presented here indicate, immunization with L144/R147 in CFA can also induce regulatory T cells of a Th2/Th0 phenotype which transfer protection even to unrelated myelin antigens (MBP and MOG). It has previously been demonstrated that suppressor cells may have a mixed cytokine phenotype, producing both Th1 and Th2 cytokines. Whereas Th1 cytokines can inhibit Th2 cells and Th2 cytokines can antagonize Th1 cells, a cell producing both Th1 and Th2 cytokines is capable of inhibiting both Th1 and Th2 cells by the virtue of cytokines it produces (28). It is interesting to speculate that in infection with human immunodeficiency and hepatitis B viruses, which elaborate variant antagonistic epitopes (29, 30), this mechanism may contribute to chronic infection both by antagonizing virus-specific T cells and also by causing immune deviation toward a Th2 phenotype which will further inhibit the generation of protective Th1 cells. Using another peptide analog of PLP-

case due t immune deviation (20). Other studies have suggested that administration of altered peptides results in a decrease in the proinflammatory cytokines tumor necrosis factor α and IFN-γ and an efflux of inflammatory cells from brain lesions (31). Similar observations have been reported in an experimental model using altered peptides derived from human collagen IV (32). However, the present study brings together two important concepts: (*i*) The TCR antagonist peptides do not prevent autoimmunity solely by antagonism *in vivo*, but induce regulatory T cells. (*ii*) The regulatory T cells mediate bystander suppression and inhibit autoimmunity initiated by T cells specific for a number of autoantigens present in the target organ.

The mechanism by which altered peptides induce T cells that regulate immune responses is unknown. It is noteworthy that a predominantly Th0/Th2 response develops even after immunization with TCR antagonist peptide emulsified in CFA, which is known to strongly favor the development of a Th1 response (33). One possibility is that altered peptide ligands engage the TCRs of naive Thp cells, which would go on to be PLP-(139–151) specific, with a lower avidity that is sufficient to alter their differentiation to a Th2/Th0 pathway as has been described in TCR transgenic systems (34, 35). If this were the case then one would predict that the TCR specificity of the clones for PLP-(139–151) and L144/R147 peptide analog would be unchanged, and we find that this is not the case (M. Prabhu Das, L.B.N., and V.K.K., unpublished results). It therefore seems likely that immunization with the L144/R147 altered peptide recruits a different population of cells and that these cells are also of a different phenotype. If differences in TCR/MHC/peptide avidity are responsible for the differences in phenotype at the population level, then this predicts that the average avidity of the population of T cells which recognizes PLP-(139–151) is different from the average avidity of the population which recognizes the altered peptide. These differences could arise because the repertoire of T cells in the periphery available to respond to PLP-(139–151) is affected by mechanisms of central and peripheral tolerance to a greater extent than the repertoire of cells available to respond to altered self peptides. Another possibility is that L144/R147 selectively antagonizes encephalitogenic Th1 precursors but expands regulatory T cells directly by acting as an agonist for them. This model is consistent with the differences we have observed between encephalitogenic Th1 and regulatory T cells (M. Prabhu Das, L.B.N., and V.K.K., unpublished results).

The demonstration that bystander suppression induced with a peptide antagonist is effective in ameliorating disease caused by structurally unrelated myelin antigens argues for its therapeutic importance. Clearly, if protective immunization can be established with a single TCR antagonist peptide which itself is nonpathogenic, antagonizes specific pathogenic Th1 cells, and induces regulatory T cells, it may provide a desirable therapeutic option for use in the treatment of many autoimmune diseases.

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A Viral Peptide with Limited Homology to a Self Peptide Can Induce Clinical Signs of Experimental Autoimmune Encephalomyelitis[1]

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Molecular mimicry has been suggested as a mode of autoreactive T cell stimulation in autoimmune diseases. Myelin basic protein (MBP) peptide 1–11 induces experimental autoimmune encephalomyelitis (EAE) in susceptible strains of mice. Here we show that a herpesvirus *Saimiri* (HVS) peptide, AAQRRPSRPFA, with a limited homology to MBP1–11 peptide, ASQKRPSQRHG (underlined letters showing homology), can stimulate a panel of MBP1–11-specific T cell hybridomas and more importantly cause EAE in mice. We demonstrate that this is due to cross-recognition of these two peptides by TCRs. Results presented in this communication are the first demonstration that a viral peptide with homology at just 5 amino acids with a self peptide can induce clinical signs of EAE in mice. These findings have important implications in understanding the breakdown of T cell tolerance to self Ags in autoimmune diseases by means of cross-reactivity with unrelated peptides. *The Journal of Immunology*, 1998, 161: 60–64.

T helper cells recognize foreign peptides bound to MHC class II molecules on APCs (1–4). In an autoimmune disease, MHC class II molecules can bind and present self peptides to pathogenic T cells (5, 6). Therefore, activation of self-reactive $CD4^+$ T cells is a crucial event in the induction of autoimmune diseases. In animals, experimental autoimmune encephalomyelitis (EAE)[3] can be transferred by T cells activated by Ags from the central nervous system (CNS) (5, 7). These cells then migrate into the CNS and cause inflammation and demyelination (5–7). However, the question remains as to the natural trigger that results in chronic activation of a self-reactive T cell repertoire in human autoimmune diseases. Environmental Ags, such as viruses and bacteria, may play a role in stimulating self-reactive T cells by a mechanism known as molecular mimicry (8). Stimulation and clonal expansion of self-reactive pathogenic T cells in the periphery can also occur by bacterial or viral superantigens (9–11). There are numerous reports that suggest viral infection may precede autoimmune diseases such as type 1 diabetes, multiple sclerosis (MS), and myocarditis (12–14). Overall, these studies suggest that the exposure to pathogens may stimulate the self-reactive T cell repertoire such that it may trigger or exacerbate autoimmunity.

MBP1–11 is a dominant epitope in MBP that induces EAE in susceptible mouse strains expressing $I\text{-}A^u$ MHC class II molecules (5, 6, 15). Previously, we and others have studied this peptide extensively to determine how it binds to $I\text{-}A^u$ class II molecules, stimulates MBP-reactive T cells clones, and induces EAE. We also determined just how much of this peptide sequence was in fact required for stimulation of T cells and for the induction of EAE (15). We showed that an 11-amino acid polyalanine peptide with just five native MBP residues could induce EAE (16).

Wucherpfennig and Strominger have provided strong evidence that peptides derived from certain viruses and bacteria could stimulate MBP-specific T cell clones generated from MS patients (17). We have utilized a similar approach of data base search based on MHC and TCR contact residues and identified a herpesvirus *Saimiri* (HVS) peptide with homology to the disease causing MBP1–11 peptide (15). This peptide stimulates MBP1–11-specific T cell hybridomas and induces clear clinical signs of EAE in some $(PL/J \times SJL/J)F_1$ mice. We show that this is due to cross-recognition between MBP1–11:$I\text{-}A^u$ and HVS peptides:$I\text{-}A^u$ complexes by $CD4^+$ T cells.

Materials and Methods

Mice

PL/J and $(PL/J \times SJL/J)F_1$ mice (8–12 wk of age) were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred at the animal facility at the John Curtin School of Medical Research (Canberra, Australia) in specific pathogen-free conditions.

Peptides

Peptides were synthesized by standard F-moc chemistry using the Applied Biosystems (Foster City, CA) model 431A peptide synthesizer. Peptides were analyzed by HPLC and purified if necessary; structure was confirmed by amino acid analysis and mass spectrometry.

Cell incubations, medium, and conditions

RPMI 1640 supplemented with either 10% FCS or 2% normal mouse serum, 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM glutamine, and 0.05 mM 2-ME was used in most cultures. Cells were incubated at 37°C in a 5% CO_2 atmosphere.

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[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; CNS, central nervous system; MBP, myelin basic protein; MS, multiple sclerosis; HVS, herpesvirus *Saimiri*.

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Lymphoproliferation assay

Popliteal lymph nodes were removed 10 days after initial immunization of mice with MBP1–11 emulsified in CFA with 400 μg of heat-killed *Mycobacterium tuberculosis* H37 Ra (Difco, Detroit, MI). Single-cell suspensions were prepared, and cells (2×10^5) were cultured in flat-bottom 96-well microtiter plates in 2% normal mouse serum with or without peptides for 72 h, pulsed with [^{3}H]thymidine, harvested, and counted as described (7). Data are presented as stimulation index on triplicate wells. The SDs were 1–15% of the mean.

T cell hybridoma assays

The T cell hybridomas were established from clones PJR-35, PJPR7.5, PJB20, and BR4, as described previously (6). Activation of T cell hybridomas (2×10^5) was assessed by incubating cells with an I-A^u-expressing B cell line (2×10^5) as APC and various concentrations of peptides. After 24 h, 50 μl of supernatant were harvested from each well and tested for IL-2 production by using an IL-2-dependent cell line HT-2, as described previously (16).

Computer modeling

A structural model of I-A^u has previously been described (18) and was obtained from the author. Initial models for MBP and HVS peptides were constructed using all four nonoverlapped alignments of the 11-residue peptide sequences with the 15-residue class II-associated invariant chain peptide (CLIP). These preliminary models were then scanned for general complementarity between residues of the peptide and the class II molecules as well as their potential to explain observed experimental data. For both the MBP and HVS peptides, the third binding mode was selected, and these models were subjected to further refinement as follows. First, the models were minimized, then shaken with a short burst of molecular dynamics, and then further minimized, making use of the DISCOVER program in the Biosym package (Biosym, San Diego, CA). Structures were initially minimized with respect to the consistent valence force field (CVFF) energy potential in vacuum for 100 steps, using steepest descent minimization, with no cross or morse terms but including charges, a distance-dependent dielectric constant, and a nonbound interaction cutoff of 12 Å. Final minimization was performed until the maximum derivative converged to 0.05 Kcal/mol Å. Molecular dynamics was used to shake the system and was performed for 100 cycles at 303 °K, allowing 1000 steps for equilibration, with all other conditions as for minimization. The final structures are shown in Figure 3.

EAE induction

EAE was induced as described (6, 15, 16) by injecting 200 μg MBP or HVS peptides emulsified in CFA s.c. at the base of the tail in a total volume of 0.1 ml. Two hundred nanograms of pertussis toxin (*JRH* Biosciences, Woodland, CA) was injected i.v. at the time of immunization and again 48 h later. Mice were examined daily for 30 to 40 days and were scored as follows: 1, limp tail; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, hind and fore limb paralysis; 5, moribund. The data are presented as cumulative incidence, calculated as total number of mice that showed signs of EAE at any point during the experiment.

Results

A viral peptide activates I-A^u-restricted MBP-specific T cell hybridomas

We have shown that a polyalanine peptide with a Gln-Lys-Arg-Pro (QKRP) motif at positions 3, 4, 5, and 6 can bind I-A^u, stimulate MBP-reactive T cells, and, most importantly, induce EAE in mice (16). Wraith and coworkers had previously identified Gln at position 3 and Pro at position 6 to be the main TCR contact residues of MBP1–11 peptide, while Lys at position 4 to be an MHC contact residue (6). The finding that a specific in vivo response can be generated by a peptide containing only five native MBP residues provided evidence that TCR from disease-inducing T cells, at least in this case, recognized only a few residues of the MHC-bound antigenic peptides. Since only five native residues in a peptide are sufficient to induce EAE, it is conceivable that a pathogen with homology to self proteins at only a few residues may trigger autoimmune disease. We therefore identified a few closely matched peptides of viral origin (Ref. 15 and Table I) and used one of them as a model unrelated viral peptide to test the hypothesis that a viral peptide with little homology with MBP1–11 can stimulate self-reactive T cells and cause EAE.

Table I. *Sequence similarities between MBP1–11 and viral proteins*[a]

Sequence	Source
AS**QKRP**SQRHG	Myelin basic protein (MBP) 1–11
AAG**RAP**ERPPR	Herpesvirus *Saimiri* (HVS) 52-kD early phosphoprotein
AAG**TRP**EKAYA	Shaklee virus × DNA
DD**GRAP**KSQNE	Herpes simplex virus (HSV) type 1
RT**QKRP**FK/HRE	Human herpesvirus 6 immediate-early gene homology
DG**KRP**VHKFL	Infectious laryngotracheitis virus (ILTV) gene
TT**QKRP**KGVPG	Sindbis virus structural polyprotein gene
LL**GKRP**SKQEP	Avian retrovirus JC proviral genome
GY**QKRP**RNRRL	Reticuloendotheliosis virus strain A
PL**GKRP**RTNAF	African swine fever virus
DS**QKRP**SSCTS	Uukuniemi virus L RNA segment for RNA polymera
QG**QKRP**SAIIL	Cabbage leaf curl virus coat protein (AVI)
SP**QKRP**TRSDH	Equine herpesvirus 1 complete genome
EP**QKRP**CHRSA	Mouse cytomegalovirus 1
PR**QKRP**KRRSI	Human foamy virus proviral DNA
EK**QKRP**ITHVP	Kaposi's sarcoma-associated herpesvirus
RL**QKRP**QPRGS	Herpes simplex virus (HSV) type 2
FP**QKRP**ARSTY	Human rhinovirus type 14 (HRV14)
HK**QKRP**WRTLS	Hepatitis C virus
IS**QKRP**SMALL	Coxsackievirus B5
TY**QKRP**IPHFY	Wheat spindel streak mosaic virus
KN**QKRP**TSRER	Cymbidium mosaic virus
QG**QKRP**SYNI	Texas pepper virus strain Tamaulipas component
TV**QKRP**CFY	Influenza A/Equine Prague/1/56 (H7N7), PB2
AL**QKRP**ELPFK	Human immunodeficiency virus type 1
AK**QKRP**PRLRT	Barely mild mosaic virus genomic RNA1 for polyp

[a] Results of a protein data base search. Similar sequences from viruses are shown. A standard search in GenBank was conducted, using 11 amino acids as XX-QKRPXXXXX (X represents any amino acid) and the FAST method of Pearson and Lipman (20).

Four MBP1–11 specific T cell hybridomas, 1934.4 (6, 16, 18), PJPR7.5, PJB20, and BR4 were used in these studies. All four hybridomas respond to MBP1–11 (Fig. 1). A peptide from HVS, identified in a computer database search as a close match with MBP1–11, was acetylated and used to examined the ability of this peptide to stimulate MBP-reactive T cells. Figure 1*B* shows that the HVS.1 peptide was able to stimulate all four T cell hybridomas but not as well as the wild-type MBP1–11 peptide. Even a short HVS peptide of 8 amino acids stimulated MBP-reactive T cell hybridoma (Fig. 1*C*). These results indicate that a cross-reactive viral peptide can stimulate self-reactive T cells.

Cross-reactivity between MBP1–11 and HVS peptides using T cells generated in vivo

If molecular mimicry is one of the mechanisms implicated in autoimmune diseases, one would predict that immunization with a homologous nonself peptide can generate a sufficient number of T cells that can be stimulated by the self peptide. We examined this by immunizing two sets of (PL/J × SJL/J)F_1 mice with either HVS.1 or MBP1–11 peptides. T cells from both groups were then stimulated in vitro by either HVS or MBP1–11 peptides (Fig. 2). These results show that lymph node cells from MBP1–11-immunized mice can be stimulated by the HVS.1 peptide. However, three- to fourfold higher concentrations of HVS.1 peptide were required for the maximum proliferation. Similarly, lymph node cells from mice immunized with HVS peptide responded to MBP1–11, but overall these responses were lower than the stimulation by HVS peptide (Fig. 2*B*). These results show clearly that

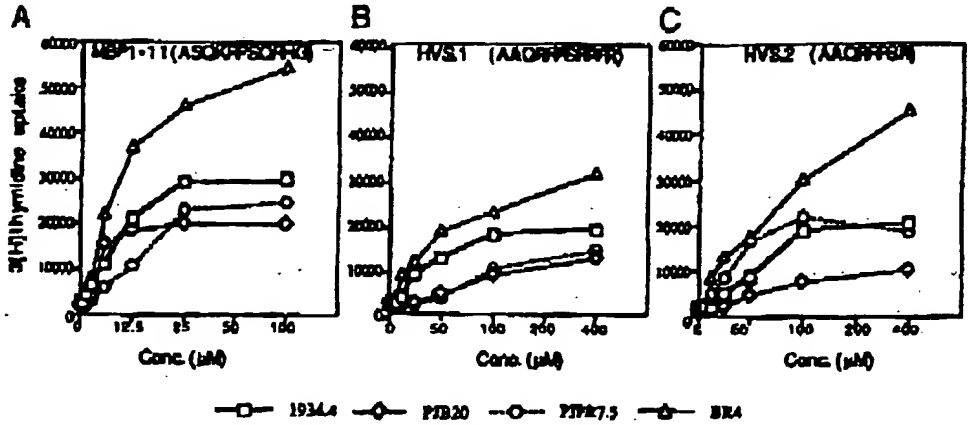

FIGURE 1. Stimulation of MBP1–11-specific T cell hybridomas by MBP1–11 and long or short HVS peptides. A total of 2×10^5 T cell hybridoma cells were incubated for 18 to 24 h with the indicated doses of peptides and an I-A^u-expressing cell line as APC. Culture supernatants were analyzed for IL-2 using IL-2-dependent cell line, HT-2, as described in *Materials and Methods*. Bold letters indicate differences between MBP1–11 and HVS peptides.

a large number of T cells can be generated in vivo that can cross-react with unrelated peptides that have some structural similarity with the immunizing peptide. Indeed, the computer modeling studies have suggested that MBP and HVS peptides are held in the groove by a similar network of hydrogen bonds, including both main chain and side chain atoms of the peptide and I-A^u molecule (Fig. 3). The major differences between MBP (Fig. 3*A*) and HVS (Fig. 3*B*) peptides are confined to the C-terminal region of the peptides. Strikingly, the backbone orientation of Arg^{P8} is such that the side chain is projected into the ridge made by the irregularity of the β-domain helix. Moreover, Pro^{P9} is constrained to the main chain with an angle of −60 degrees and Phe^{P10} must be oriented in such a way as to occupy $Asp^{\alpha 72}$, $Leu^{\alpha 77}$, $Tyr^{\beta 30}$, and $Tyr^{\beta 37}$ pocket. These differences may explain slightly different patterns of immune responses initiated by the HVS peptides compare to MBP1–11 peptide (See Figs. 1, 2, and 4).

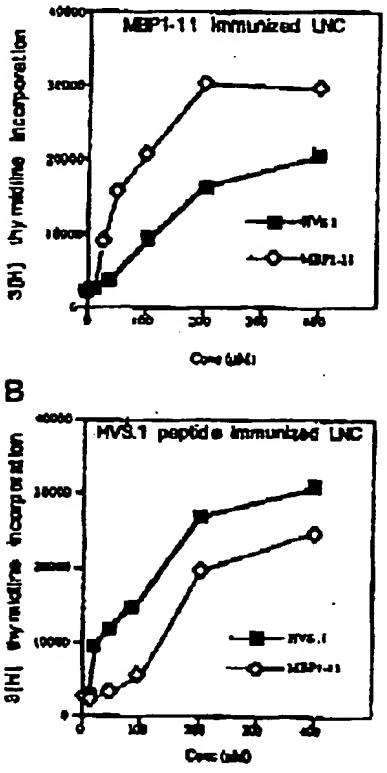

FIGURE 2. Cross-sensitization of MBP1–11-reactive T cells by HVS peptide. Mice were immunized either with 100 μg of MBP1–11 or with HVS.1 peptides in CFA. Nine days post-immunization, lymph nodes were removed and proliferated with the indicated doses of peptides for 3 days. Cells were harvested after [^{3}H]thymidine incorporation as described in *Materials and Methods*. The SDs ranged between 10 and 18 in this experiment.

The cross-reactive viral peptides induce clinical and histologic signs of EAE

The most challenging test to address cross-reactivity and molecular mimicry is to assess whether HVS peptides can induce EAE. To

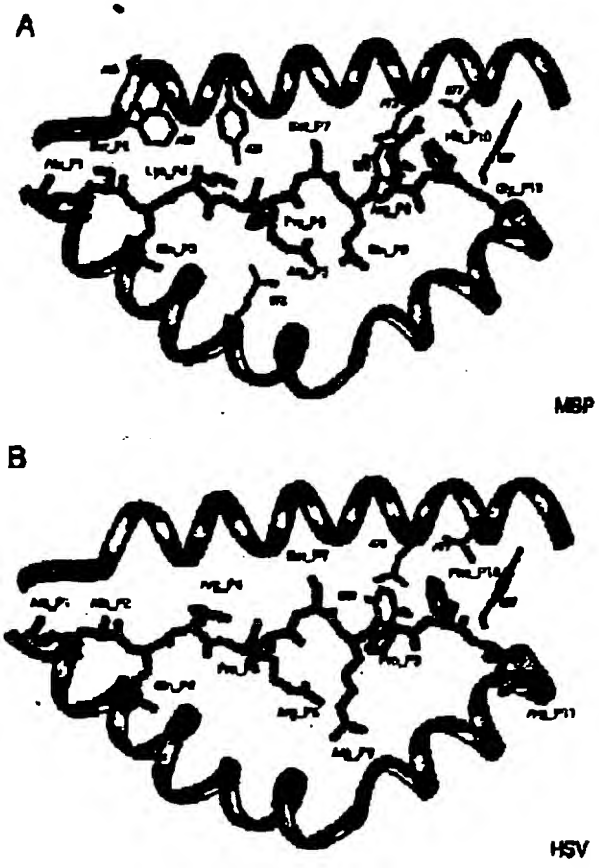

FIGURE 3. Computer models of MBP (*A*) and HSV (*B*) peptides with I-A^u MHC class II molecules as described in *Materials and Methods*.

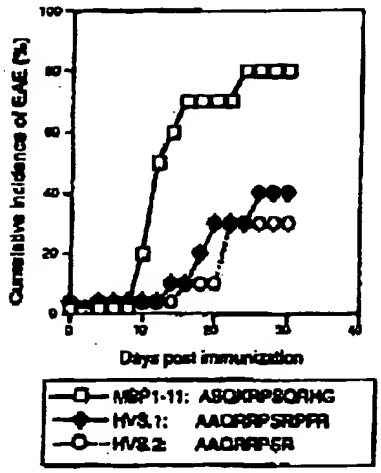

FIGURE 4. HVS peptides induce EAE. EAE in (PL/J × SJL/J) was induced as described in *Materials and Methods*. Peptides (200 μg) emulsified in CFA with 400 μg *Mycobacterium tuberculosis* were injected s.c. at the base of the tails in a total volume of 100 μl for each peptide. Mice were examined daily for 30 days. A cumulative incidence of EAE is shown. At least 10 mice were used in each group.

test this, we immunized (PL/J × SJL/J)F_1 mice with either HVS or MBP1–11 peptides to induce EAE. Figure 4 shows that up to 40% of HVS.1 peptide-immunized mice developed EAE albeit with a slightly reduced severity. While MBP-immunized mice had a mean clinical score of 3.5, the HVS.1 peptide-immunized mice had a mean clinical score of 2.5. Even a short 8-amino acid HVS.2 peptide can induce EAE in 20 to 30% of mice. EAE in these mice was associated with typical perivascular cellular infiltrations in the spinal cord (Fig. 5*b*). The mice that did not develop clear clinical signs of EAE showed some cellular infiltration in the CNS (data not shown). Immunization of control mice with peptides with no sequence homology with MBP1–11 (e.g., ova323–339 or MBP89–101) have never resulted in any clinical or histologic signs of EAE (data not shown).

Discussion

In this study we have provided the first clear demonstration that EAE in mice can be induced by a cross-reactive viral peptide. Although histologic signs of EAE in rabbits were previously demonstrated by Fujinami and Oldstone (19), to our knowledge there is no report showing induction of the clinical signs of EAE by a cross-reactive microbial peptide. Indeed, Fujinami and Oldstone stated clearly that none of the rabbits immunized with hepatitis B virus polymerase peptide (HBVP) develop clinical signs of EAE with the protocol used. We know from our own studies that cellular infiltration in the CNS does not always result in a subsequent clinical disease. Therefore, our demonstration that a viral peptide could induce clear clinical signs of EAE not only complements the previous work by Oldstone but also provides credence for the molecular mimicry model. In addition, two groups have recently shown that structurally similar peptides (17) and random peptide libraries (20) could stimulate MBP-reactive T cells clones. Both studies hypothesize that a similar mechanism may operate in stimulating self-reactive T cells in individuals leading to tissue damage and autoimmune disease. This study provides experimental support for this hypothesis.

We have shown previously that a polyalanine peptide with only a few native MBP amino acids, Gln-Lys-Arg-Pro (QKRP), could induce EAE with a severity identical to that of MBP1–11 (16). This led us to propose that a microbial peptide with some similarity with MBP1–11 could stimulate MBP-reactive T cells and

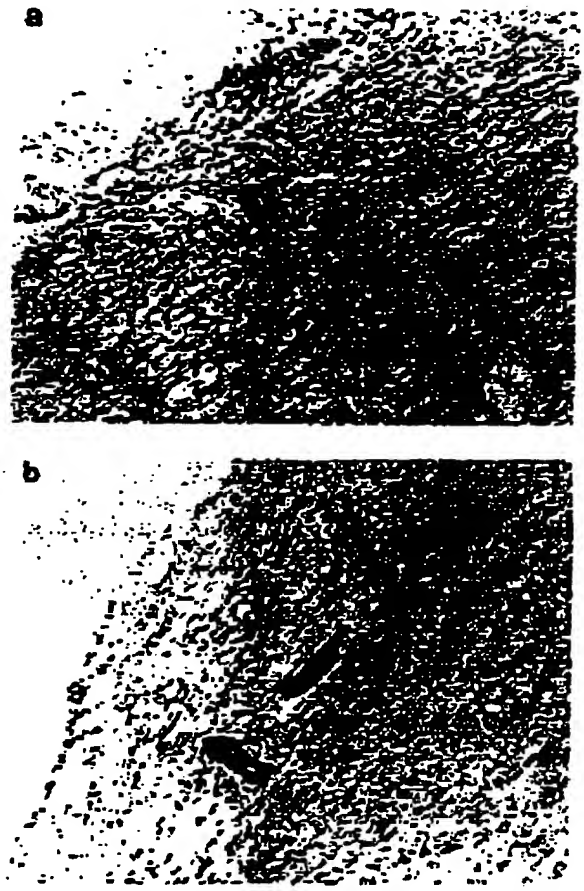

FIGURE 5. Representative perivascular cellular infiltration in the spinal cord sections of mice immunized with either MBP1–11 or HVS-1 peptides. Longitudinal thin sections of cervical spinal cord were cut 16 days postimmunization with peptides and stained with hematoxylin and eosin stain. *a*, MBP1–11- and (*b*) HVS-1-immunized mouse.

cause EAE (15). The HVS peptide (AAQRRPSRPFR) selected in this study is radically different from MBP1–11, especially on the C-terminal end. In HVS peptide, even the core sequence differs by 1 amino acid (Lys to Arg at position 4). McDevitt and colleagues have shown previously that there is a dramatic change in the binding properties of MBP1–11 peptide to $I\text{-}A^u$ if position 4 is substituted with certain amino acids (6, 16). It is also important to note that His at position 10 in MBP1–11 improves binding of this peptide to $I\text{-}A^u$ and enhances induction of EAE (16). Another major difference between MBP1–11 and HVS peptides is the addition of a Proline residue at position 9. This may lead to a "kink" in the peptide. As shown here, the incidence of EAE by HVS peptides is much reduced compared with MBP1–11. This could be due to their ability to stimulate only a subset of MBP1–11-specific T cells, and/or the HVS peptides may act as weak or partial agonist for MBP-reactive TCRs.

Four mechanisms have been proposed to explain the activation of an autoimmune process by infections. The first is molecular mimicry, implying some level of homology between a self Ag and an infectious agent (8, 19, 21–23). This mechanism can operate for both Ab- and T cell-mediated autoimmune diseases (8, 21–24). The second is activation of a subset of T cells containing self-reactive lymphocytes by a bacterial or viral superantigen. Experimental evidence for such a mechanism exists (9), and indication that such a mechanism could be at play in a human autoimmune disease has recently been published (11). These two mechanisms do not require that the infection take place in the tissue that would be a target of the subsequent autoimmune disease. Thirdly, infection in a tissue may favor the release of

self Ags that can be processed and presented to self-reactive T cells leading to the tissue damage. Evidence for such a mechanism has recently been provided in the Theiler's virus encephalomyelitis (25). Finally, viral-specific T cells in a tissue may activate bystander self-reactive T cells (26). This mechanism has not yet been worked out at the molecular level but appears attractive in view of the fact that cytokine combinations can activate both naive and memory T cells.

How would microbial pathogens initiate an autoimmune disease? One possibility is that self-reactive T cells that have escaped the thymic deletion encounter cross-reactive microbial peptides bound to MHC molecules. This may result in the low level stimulation of self-reactive T cells, migration into a site, and the causing of some tissue damage. Once the tissue destruction has begun, the release of self Ag from the target tissue may perpetuate the immune response against its own Ags even after the microbial pathogen has been cleared. It is important to note that, at least in our model of EAE, MBP1–11 peptide binds I-A^u poorly (6, 16). This poor binding of MBP1–11 could result in an inefficient negative T cell selection for this peptide in the thymus. Once in periphery, these cells could be potentially autoreactive, waiting to be stimulated by a trigger such as a virus or bacteria. As has been shown by others, TCR is capable of being stimulated by a variety of peptides presented by the same MHC class II molecule (16, 17, 20, 27).

A question also arises whether a similar viral peptide could contribute to MS pathology. Molecular mimicry has been described between MBP and several viral peptides (Refs. 12, 21, 22; for a brief review see also Refs. 8, 23). Moreover, MS-like symptoms have been observed in a series of patients following hepatitis B surface Ag vaccination (28). There is also convincing evidence for molecular mimicry between *Campylobacter jejuni* and Guillain-Barre syndrome (a peripheral nerve inflammatory demyelinating disease) (24). Clearly we need to investigate whether cross-reactive peptides can 1) be generated from microbial pathogens and 2) be loaded on to MHC molecules. There are several examples in the literature that demonstrate clearly that MHC class II molecules can present peptides derived from intracellular proteins (29–32). This would suggest that a cross-reactive peptide from an intracellular virus could in theory be loaded onto class II MHC molecules and presented to $CD4^+$ encephalitogenic T cells. Peptides derived from extracellular pathogens have not yet been well studied for stimulating self-reactive $CD4^+$ T cells. However, it is plausible that cross-reactive peptides generated from extracellular pathogens via a well-defined class II Ag-processing pathway could result in cross-recognition by autoaggressive T cells (for review on class II see Ref. 2).

In conclusion, we have presented experiments that show that a cross-reactive nonself peptide from a viral protein can generate immune response such that it could lead to a clinical autoimmune disease.

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